Pre-clinical *in vivo* studies of parenteral drug delivery systems

using non-invasive multispectral fluorescence imaging

Dissertation

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Das Schönste, was wir entdecken können, ist das Geheimnisvolle.

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein (1879 - 1955)
## Contents

### Chapter I: Introduction

**A** Pre-clinical imaging in pharmaceutical research

- The role of pre-clinical studies in drug development
- The importance of pre-clinical studies to research the in vivo behavior of APIs
- Imaging systems used for pre-clinical studies
- Potential application areas of pre-clinical fluorescence imaging

**B** Research objectives

**C** Basic principles

- Fundamentals of fluorescence imaging
  - Overall principles of optical imaging
  - Principles of fluorescence
  - Dyes applicable for fluorescence imaging
  - Intrinsic limitations of in vivo optical imaging
  - Challenges when using in vivo imaging
  - Measurement systems for optical in vivo imaging
- The Maestro in vivo imaging system
  - Instrumentation
  - Measurement fundamentals
  - Acquisition of multispectral fluorescence images
  - Spectral unmixing and auto-fluorescence removal
  - Analyses of measurement files

### Chapter II: Results of pre-clinical in vivo studies of polymers

**D** Long-term in vivo biodistribution studies of i.p. injected high molecular weight PVA

- Summary
Chapter III: Results of pre-clinical in vivo studies of nanoparticles 34

E In vivo characterization of nanocarriers and their potential capability in cancer therapies.................................................................................................................. 34
E 1. Summary................................................................................................... 34

F Investigating the potential toxicity risk of nanocarriers ........................................ 37
F 1. Summary................................................................................................... 37

Chapter IV: Results of pre-clinical in vivo studies of in situ forming implants 39

G Long-term in vivo pH measurements of in situ forming PLGA implants .......... 39
G 1. Summary................................................................................................... 39

Chapter V: Results of alternative application fields of fluorescence imaging 41

H Monitoring internal pH gradients in swelling multi-layer tablets...................... 41
H 1. Summary................................................................................................... 41

I Monitoring temperature distributions in tablets - caused by the tableting process....................................................................................................................... 43
I 1. Summary................................................................................................... 43

J In vivo and ex vivo characterization of nanocapsules........................................ 44
J 1. Summary................................................................................................... 44

K Monitoring the in vivo efficiency of rhBMP-2 loaded microparticles ............... 45
K 1. Summary................................................................................................... 45

Chapter VI: Fluorescence pre-clinical imaging – an overall discussion of results 46

L Potential capabilities and limitations of pre-clinical fluorescence imaging ....... 46

M Major limitations of pre-clinical fluorescence imaging studies......................... 47

N Major challenges of pre-clinical fluorescence imaging studies........................ 48
N 1. The choice of the correct dye.................................................................... 48
N 2. Quantification – an impossible challenge in pre-clinical in vivo experiments? .................................................................................................................. 50

Chapter VII: Summary and future perspectives 54

O Summary............................................................................................................ 54

P Future perspectives............................................................................................ 60

Q German summary............................................................................................... 61
(a) Investigation of the in vivo fate of a water soluble polymer

(I) Noninvasive in vivo monitoring of the biofate of 195 kDa poly(vinyl alcohol) by multispectral fluorescence imaging.

(II) In-vivo studies on intraperitoneally administrated poly(vinyl alcohol).

(III) Tracking the in vivo fate of high molar mass poly(vinyl alcohol) using multispectral fluorescence in vivo imaging.

(b) Characterization of nanocarriers and their potential usage in cancer therapy

(IV) How stealthy are PEG-PLA nanoparticles? An NIR in vivo study combined with detailed size measurements.

(V) Tumor accumulation of NIR fluorescent PEG PLA nanoparticles: impact of particle size and human xenograft tumor model.

(VI) Accumulation of nanocarriers in the ovary: A neglected toxicity risk?

(c) Characterization of in situ forming implants for potential controlled API release


(d) Investigating alternative application fields of fluorescence imaging

(VIII) Monitoring of internal pH gradients within multi-layer tablets by optical methods and EPR imaging.

Annex

Acknowledgements ........................................................................................................ I
Publication list............................................................................................................... III
Curriculum vitae........................................................................................................... VII
Declaration of the self-contribution of research articles.............................................. VIII
Selbstständigkeitserklärung........................................................................................ XI
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF4</td>
<td>Asymmetrical Flow Field Flow Fractionation</td>
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<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
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<tr>
<td>BCS</td>
<td>Biopharmaceutical Classification System</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>CCD</td>
<td>Charged-Coupled Device</td>
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<tr>
<td>Cri</td>
<td>Cambridge research &amp; instrumentation</td>
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<tr>
<td>CT</td>
<td>x-ray Computed Tomography</td>
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<td>Cy</td>
<td>Cyanine</td>
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<td>Da</td>
<td>Dalton</td>
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<td>Di</td>
<td>Dialkylcarbocyanine</td>
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<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
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<td>DOT</td>
<td>Diffuse Optical Tomography</td>
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<tr>
<td>Emax</td>
<td>Emission maximum</td>
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<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance (in German ESR)</td>
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<td>ESR</td>
<td>Elektronenspinresonanz (in English EPR)</td>
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<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
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<tr>
<td>FITC</td>
<td>Fluorescein IsoThioCyanate</td>
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<td>FMT</td>
<td>Fluorescence-Mediated Tomography</td>
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<tr>
<td>i.m.</td>
<td><em>intramuscular</em></td>
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<tr>
<td>i.p.</td>
<td><em>intraperitoneal</em></td>
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<tr>
<td>i.v.</td>
<td><em>intravenous</em></td>
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<tr>
<td>IR</td>
<td>InfraRed</td>
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<tr>
<td>LED</td>
<td>Light-Emitting Diode</td>
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<tr>
<td>MALLS</td>
<td>MultiAngle Laser Light Scattering</td>
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<td>MPS</td>
<td>Mononuclear Phagocytic System</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>NIR</td>
<td>Near InfraRed</td>
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<tr>
<td>NR</td>
<td>Nile Red</td>
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<tr>
<td>OPT</td>
<td>Optical Projection Tomography</td>
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<tr>
<td>PCS</td>
<td>Photon Correlation Spectroscopy</td>
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<td>PDI</td>
<td>PolyDispersity Index</td>
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<tr>
<td>PEG</td>
<td>PolyEthylene Glycol</td>
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<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PLA</td>
<td>PolyLactic Acid</td>
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<tr>
<td>PLGA</td>
<td>Poly(Lactic-Co-Glycolic) Acid</td>
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<tr>
<td>PVA</td>
<td>Poly(Vinyl Alcohol)</td>
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<tr>
<td>QD</td>
<td>Quantum Dots</td>
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<tr>
<td>RGB</td>
<td>Red-Green-Blue</td>
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<tr>
<td>rh</td>
<td>recombinant human</td>
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<tr>
<td>RNA</td>
<td>RiboNucleic Acid</td>
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<tr>
<td>ROI</td>
<td>Region Of Interest</td>
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<tr>
<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>SNARF</td>
<td>SemiNaphthoRhodaFluor</td>
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<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>TMR</td>
<td>TetraMethylRhodamine</td>
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<td>VIS</td>
<td>VISible</td>
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Preface

This dissertation is prepared as a cumulative PhD Thesis. The results of the pre-clinical *in vivo* studies of parenteral drug delivery systems using non-invasive multispectral fluorescence imaging are discussed in several publications. Those are already published or submitted for publication. They contain the results obtained during my research in the Pharmaceutical Technology Group (Faculty of Pharmacy) at the Martin Luther University Halle-Wittenberg, Germany. The PhD Thesis was prepared under the supervision of Professor Dr. rer. nat. habil. Karsten Mäder. The respective chapters within this dissertation are supposed to be a summary of the corresponding publications [1-8] which can be found in the ‘Supplemental Material’ section of this dissertation.

Namely, this cumulative PhD Thesis consists of the following research articles which are grouped into four research topics (a-d):

(a) Investigation of the *in vivo* fate of a water soluble polymer


(b) Characterization of nanocarriers and their potential usage in cancer therapy


(c) Characterization of in situ forming implants for potential controlled API release

(VII) Schädlich, A., Kempe, S., Mäder, K., (2013) Long-term in vivo pH measurements of in situ forming PLGA implants using multispectral fluorescence imaging. Submitted to Journal of Controlled Release (under peer-review) [7].

(d) Investigating alternative application fields of fluorescence imaging

Chapter I: Introduction

A Pre-clinical imaging in pharmaceutical research

A 1. The role of pre-clinical studies in drug development

The process of discovering, producing and launching new active pharmaceutical ingredients (API) is expensive and time-consuming. Several development and testing phases must be passed through, until a new pharmaceutical product is allowed to be sold on the market to treat human diseases. Thereby, many of the original promising APIs fail and never get the marketing authorization.

However, before a new API can be registered as a medicinal product, it has to be tested for its safety and efficacy in cells, animals and humans. Pre-clinical studies in animals must be conducted in order to test the pharmacology, pharmacokinetic and toxicology effects. Those are related to the API but also mainly influenced by the route of administration and the drug delivery system. As the drug formulation has a major effect on the effectiveness of the API they are often already explored within the first pre-clinical animal experiments. If these pre-clinical tests were promising three extensive and cost-intensive clinical phase studies in humans have to be followed. They sometimes lasting over a decade before the new tested API possibly reach the marketing authorization application step and consequently the market. In average this long and expensive undertaking requires often more than $800 million per new molecular entity while the development takes about 10 years to 13 years before a new drug is available on the market [9-11]. Thereby 58 % of the total costs and half of the development period are apportionable to clinical studies [10]. Within this long undertaking pre-clinical studies are milestones. As much information as possible have to be gathered based on performed animal studies in order to make the right decisions regarding the prospects of the API afterwards. However, studies in animals are complex. To use a new technique for pre-clinical studies it has to be evaluated in detail using various drug delivery systems prior it can be commonly applied in drug discovery research.
A 2. The importance of pre-clinical studies to research the *in vivo* behavior of APIs

More and more new APIs have increased potency and selectivity in their effects but do also have concomitant challenging physical properties, like high molecular masses and increased hydrophobic characteristics [12; 13]. To simply classify new APIs, the Biopharmaceutical Classification System (BCS) has become an important tool since it was introduced in 1995 [14]. Most of the new APIs can be assigned to BCS class II or IV based on their poor water solubility. Due to this property, the galenics of the API is very crucial and the formulation itself gets a pivotal influence. Knowing the complex *in vivo* behavior of the galenic formulations is important for the improvement of the drug development process. The galenic has a major influence to the therapeutic effect of an API and is essential for the future success of clinical studies. However, the API behavior in the formulation as well as the release of the API is often hardly predictable *in vitro*. This complexity demands more and more pre-clinical *in vivo* tests which increases the development costs. To reduce ethical problematic and cost-intensive pre-clinical animal experiments, new drug candidates and also the formulation approaches with the desired best pharmacological properties for further application have to be identified as early as possible.

In addition, the effectiveness of the pharmaceutical development and the prediction expressiveness of *in vitro* experiments must be increased just as well as the informative values of pre-clinical *in vivo* studies. However, even the ideal API for the respective target from a biological perspective is of little value if it is not transported to the desired site of action in the body. Therefore, the API biodistribution research is very crucial and should be conducted in complex *in vivo* surroundings. The *in vivo* imaging technique has the capability to assess the *in vivo* fate and allows answering target biological questions in early pre-clinical trials. The results can increase the efficiency of potential API candidate selection by providing earlier, more predictive information about potential *in vivo* behavior in humans.

A 3. Imaging systems used for pre-clinical studies

Several anatomical and functional imaging systems for humans have been developed over the past 30 years, including X-ray Computed Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) [15-17]. Based on their imaging modality each of these systems is used for different indications. For example
CT can visualize bone tissue and with use of certain contrast agents soft tissues. MRI offers the highest spatial resolution and best soft tissue imaging. Functional imaging methods like PET and SPECT represent high resolution and high sensitivity in nuclear functional methodologies. The PET method requires the use of isotopes and tracer molecules. But the number of isotopes which are available to be incorporated to characterize drug delivery systems is limited. SPECT, on the other hand, offers easier access to isotopes which are also suitable for the labeling of biologicals like peptides and antibodies. These techniques have an important impact to the development and therapy in modern human clinical medicine but they are very expensive in acquisition and servicing [15]. Therefore, these techniques are normally reserved to a restricted number of patients and are often not used for extensive pre-clinical tests. Hence, the interest in the affordable and powerful imaging systems for small animal models has highly increased over the last 10 years [18; 19].

An overview of available pre-clinical imaging techniques to be used for the imaging of small animals is shown in Figure 1. Due to the limitations of some techniques nowadays also combinations of two different measurement approaches within one imaging system are available on the market.

Fluorescence imaging is characterized by short measurement times and a broad variety of application fields due to hundreds of available fluorescence dyes with varied physicochemical and light emitting properties. Compared to CT, MRI, PET and SPECT the equipment is relatively inexpensive. Altogether this enables optical imaging and especially fluorescence imaging to be a powerful multifunctional tool for molecular in vivo imaging [20-22]. But there is an incomplete understanding of the
actual fundamental capabilities and limitations of fluorescence \textit{in vivo} imaging (Figure 2). Thus, this imaging technique has not yet reached a state of routine use in pre-clinical and clinical studies [23].

The purpose of this research was to identify application possibilities as well as of constraints when using fluorescence imaging in the drug delivery research field in order to improve pre-clinical studies in future. Thereby, the \textit{in vivo} behavior of different parenteral drug carrier systems should be researched in detail. The research was focused on key issues required for the pre-clinical characterization of parenteral formulations like the feasibility of measuring \textit{in vivo} compound distribution, accumulation and elimination processes as well as release profiles of incorporated dyes and measuring the pH \textit{in vivo}. In addition a variety of topics (illustrated in Figure 2) had to be evaluated.

![Figure 2: Variety of topics to be researched when using fluorescence imaging in pre-clinical experiments.](image)

**A 4. Potential application areas of pre-clinical fluorescence imaging**

To explore the fluorescence imaging extensively, three different parenteral drug delivery systems, (a) water soluble polymer conjugates (publication I - III), (b) nanocarriers (publication IV - VI) and (c) \textit{in situ} forming implants (publication VII), with expected complex \textit{in vivo} behaviors were chosen and injected via three different administration routes: (a) intraperitoneal (\textit{i.p.}), (b) intravenously (\textit{i.v.}) and (c)
subcutaneously \((s.c.)\) [1-7]. The gained knowledge from the performed pre-clinical \(in\ viva\) studies should enable to identify also (d) alternative application fields for fluorescence imaging within the pharmaceutical research field (publication VIII) [8].

**a) Investigation of the \(in\ viva\) fate of a water soluble polymer**

The knowledge about the \(in\ viva\) distribution pathway of polymers is essential for their further administration in humans. A high molecular weight (195 kDa) poly(vinyl alcohol), PVA was chosen to track body distribution, accumulation and elimination processes of polymers using pre-clinical imaging. The PVA polymer is relatively inert in body fluids. PVA formulations are approved by the Food and Drug Administration (FDA) and are already used in various pharmaceutical and biomedical applications [24]. PVA is further applied in biotechnology and food chemistry [24-28]. Due to its excellent film-forming, emulsifying and adhesive characteristics, PVA hydro-gels are researched for accelerating wound healing and preventing postoperative adhesions [29-33]. It is already known that high molecular weight PVA remains at least 7 to 10 days at the abdominal wall when the gel is applied over the wound field [34]. The first \(in\ viva\) studies indicated an elimination of PVA via the kidneys despite the high molecular weight [34]. However the detailed \(in\ viva\) fate after \(i.p.\) application as well the possible long-term PVA accumulations were still unexplored. It should be investigated if fluorescence imaging can be used for \(in\ viva\) and additional \(ex\ viva\) studies to identify the complex \(in\ viva\) fate of PVA. In addition the requirements concerning the choice of dyes, the measurement parameters and possible dye quantification \(in\ viva\) and also \(ex\ viva\) should be investigated.

**b) Characterization of nanocarriers and their potential usage in cancer therapy**

In anticancer therapies commonly used chemotherapeutic agents are known to be generally distributed non-specifically in the body. Thereby, they simultaneously affect both cancerous- and normal, healthy cells. However, they are therapeutically inefficient if the API is not able to access its site of action. Nanocarriers can enhance the intracellular concentration of drugs in cancer cells while avoiding toxicity in healthy cells [35; 36]. Nanocarriers like quantum dots (QD) are already used for the fluorescence tissue imaging [37-39]. Nevertheless QDs are criticized for their high production costs, potential \(in\ viva\) toxicity depending on used materials as well as their surface properties [40] and also their limited drug loading capacities. Nanoparticles can be classified into nanocapsules and nanospheres. Compared to QDs they enable an extended drug loading. Nanocapsules are colloidal-sized and
consist of an external polymer layer and an inner core serving as a reservoir that
normally represents an oily or aqueous phase which is able to encapsulate the drug
molecules [41-45]. Nanospheres can be described as a matrix-type, solid, colloidal
particle in which the drug molecules are molecularly dispersed, entrapped,
chemically bound or adsorbed to the constituent polymer matrix [46; 47]. The
industrial development of both types of nanocarriers is amongst others limited by
stability problems of their suspensions [48]. Sedimentation and aggregation
processes of the nanocarriers occur very slowly due to mixing tendencies of diffusion
and convection of the nanometer sized particles [49]. However, after several months
of storage in aqueous environment, aggregation can occur next to microbiological
growth, hydrolysis of the polymer and drug leakage [49-51]. Freeze drying can avoid
this but it is challenging in the case of nanocapsules which can often not be
lyophilized due to low stability properties. As result of their vesicular character they
tend to collapse during the procedure [51]. Nanospheres can more easily be
transferred into the dry state and are stable over months [52]. This increases their
potential use in future medicinal therapies. Therefore, nanospheres were chosen
within this research as a model nanocarrier system to investigate the size dependent
in vivo fate as well as size related tumor accumulation characteristics using pre-
clinical fluorescence imaging. In this work the nanospheres are to be further referred
as nanoparticles.
Polylactic acid (PLA) and poly(lactic-co-glycolic) acid (PLGA) are well known
hydrophobic polymer matrices, which are good suitable for the incorporation of
poorly-water soluble APIs. Both are approved by the FDA in several market products
for parenteral application and are frequently used for the purpose of nanoparticle
production [53]. PLA is the more hydrophobic polymer and therefore best suitable as
biodegradable and biocompatible one which allows the reproducible production of
homogeneous nanoparticles [53]. Besides the size also surface properties of
nanoparticles play an important role for their in vivo fate. It is well established that
hydrophilic surfaces, (e.g. achieved by polymer modification with polyethylene glycol,
PEG) reduces opsonisation and through it an uptake by the mononuclear phagocytic
system (MPS) [54; 55]. Such PEG shells also provide a sterical stabilization of the
particles in aqueous systems [56]. Based on this knowledge, PEG-PLA block
polymers were synthesized in the group of Prof. Dr. habil. A. Göpferich in
Regensburg and were used for the production of defined, stable and nontoxic
nanoparticles varying in size [52]. It is well known that body distribution, elimination
processes and pathways as well as tumor accumulation behavior of nanoparticles
are strongly particle size dependent [55; 57-62]. But there is a lack of knowledge about the exact size dependent in vivo behavior and the tumor accumulation capability of nanoparticles. Based on published in vivo studies, with polydispersities of nanocarriers between 0.2 and 0.5 [63-65], statements about particle size dependencies are doubtful. An overlapping of particle sizing can be presumed due to the broad distributions of particle sizes.

To study the in vivo biodistribution and the tumor accumulation particularly, considering potential size dependencies, comprehensive in vitro characterizations are a prerequisite to ensure the use of well-defined nanoparticles with narrow sized distributions. Size characterizations and stability studies of nanoparticle dispersions, together with detailed dye suitability studies must be prefixed within this research topic before statements regarding pre-clinical in vivo studies can be made. To investigate biodistribution in vivo, calculation methods to quantify and compare several nanoparticle batches based on detected fluorescence intensities have to be established. This is hampered by different absorption, scattering and auto-fluorescence effects of miscellaneous tissues. It is also influenced by the properties of the used dye, the light emission spectra as well as the stability and the quenching effects of the dye. Furthermore, the emission position in the body plays a crucial role. These influencing factors have to be considered in detail when using fluorescence imaging for nanocarrier characterization in pre-clinical studies in future.

Based on the obtained knowledge detailed pre-clinical tumor accumulation studies should be followed within this research to identify in which extend quantifications and size dependent predictions of the tumor accumulation behavior of nanoparticles can be made using fluorescence imaging.

(c) Characterization of in situ forming implants for potential controlled API release

In situ forming drug delivery systems are innovative formulation types which facilitate a controlled release of APIs over days up to months. They can easily be injected by a syringe into the target site. Among plenty of investigated synthetic and natural polymers, biodegradable PLA and PLGA polymers are most widespread used for the implant preparation. The popularity is based on the fact that these polymers are well characterized and provide an excellent safety and biocompatibility record [66-68]. PLGA for instance, has already been applied in some commercially available parenteral applied controlled releasing products like Eligard, Sandostatin LAR, Lupron Depot, Decapeptyl SR, Suprecur MP, Risperdal Consta and Atridox [69-72].
However, the formulation and release of proteins and peptides as well as of pH dependent poorly water soluble drugs appeared to be very difficult when PLGA systems are used. The degradation products of PLGA are acidic in nature [73]. This can cause protein instabilities, changes in API solubility and API decomposition during manufacturing, storage and application processes [68; 73-75]. Accelerated polymer degradation prior to API release could occur due to low pH values. *In vitro* experiments with PLGA microspheres showed that during incubation under physiologic conditions the pH value in the microspheres can drop from pH 7.4 to values around pH 3 [76; 77]. Although, the pH behavior of PLGA systems is intensively researched *in vitro*, only very few *in vivo* data is available [78]. Under *in vivo* conditions complex factors like perfusion, body liquids, enzymes, elimination processes may also strongly influence the pH in PLGA implants. Until now, the possibilities of fluorescence pH measurements are restricted to confocal microscopy studies measuring for example the microclimate in microspheres [77; 79]. These measurement principles are not by default transferable to the *in vivo* studies and are limited to the skin surface [80]. However, measurements of pH values in *in vivo* studies using non-invasive fluorescence imaging is hampered by a multitude of influencing factors like auto-fluorescence of skin, lack of capable dyes with sufficient quantum yields, wavelength dependent light absorption, intensity variations, possible bleaching and many others. Those factors have to be thoroughly characterized *in vitro* and *ex vivo* prior to *in vivo* measurements.

**(d) Investigating alternative application fields of fluorescence imaging**

This work was focused on the research of the *in vivo* behavior of PVA, nanoparticles and *in situ* forming PLGA implants. Nevertheless, the obtained knowledge should also be used to characterize other drug delivery systems. Therefore, cooperation projects to transfer established measurement and analyzing techniques to other comparable and also alternative formulation systems were initiated within this research project.
B Research objectives

Due to fast growing market demands for complex APIs like peptides, monoclonal antibodies and highly lipophilic drugs, parenteral applications have attracted increasing scientific and commercial attention over the last decade. Therewith, also the need of parenteral controlled delivery formulations and of targeted carriers arose. While pre-clinical animal studies and clinical studies in humans are ethnic problematic, time and cost-intensive, in vitro characterization techniques often not yield in predictable in vivo results. This makes it necessary to establish reliable pre-clinical methods to research complex in vivo behaviors. New pre-clinical non-invasive fluorescence imaging systems were launched in the early twenty-first century. But there is a lack of data available in which extension that technique can be applied to characterize parenteral formulations in vivo.

The current work focuses on the in vivo and ex vivo characterization of parenteral formulations by multispectral fluorescence imaging. Key measurements within these studies are exemplarily shown in Figure 3.

Figure 3: Overview chart of key measurement issues within the current work while using fluorescence imaging.
Briefly, the research objectives of the present cumulative thesis were focused on the use of non-invasive, multispectral fluorescence imaging in pre-clinical in vivo studies as an analytical tool for the investigation of:

(a) **Investigation of the in vivo fate of a water soluble polymer**
- Researching the in vivo fate of poly(vinyl alcohol) with focus on the accumulation as well as of elimination processes.
- Investigating analyzing methods for the characterization of release profiles and of gender specific variations

(b) **Characterization of nanocarriers and their potential usage in cancer therapy**
- Exploring methods for size dependent in vivo distribution, accumulation and elimination studies of PEG-PLA nanoparticles.
- Researching the size dependent in vivo tumor accumulation using NIR fluorescent PEG-PLA nanoparticles and fluorescent tumor xenografts.
- Studying the potential in vivo toxicity of nanocarriers.

(c) **Characterization of in situ forming implants for potential controlled API release**
- Investigating in vivo measurement approaches to detect microclimate pH values inside in situ forming PLGA implants.

(d) **Investigating alternative application fields of fluorescence imaging**
- Transfer the obtained fluorescence imaging method and analyzing knowledge to other formulation systems and pharmaceutical technology research fields.
- Monitoring of internal pH gradients in swelling multi-layer tablets.
- Monitoring of temperature distributions in tablets during tableting process.
- Measurement of the in vivo biodistribution of nanocapsules.
The appropriate characterization of parenteral formulations using fluorescence imaging is highly challenging. In order to obtain and discuss meaningful in vivo results complementary in vivo, ex vivo and in vitro measurement methods were applied (Figure 4). Detailed analytical in vitro measurements were additionally performed prior the in vivo application of the drug delivery systems to ensure the required pharmaceutical quality (Figure 4).

Figure 4: Overview chart of complementary in vivo, ex vivo and in vitro measurement methods applied for the characterization of drug delivery systems.
C  Basic principles

C 1. Fundamentals of fluorescence imaging

C 1.1. Overall principles of optical imaging
Clinical imaging systems for humans, like MRI, CT, PET and SPECT are primarily used for displaying anatomical, physiological, and metabolic parameters [15-17]. Additionally first smaller, experimental imaging systems have been developed which allow to research also cellular and molecular levels in vivo non-invasively in animals [81-83]. Optical imaging is the most often used single pre-clinical imaging modality followed by MRI and PET [84]. Currently, various optical imaging systems are available on the market. They differ in spatial resolution, sensitivity, for image generation expended energy and in resulting penetration measurement depths [23; 85; 86]. The properties are mainly influenced by the used electromagnetic spectrum. The spectra ranges of selected techniques are shown in Figure 5.

![Electromagnetic spectra and respective application techniques. Figure adapted according to Hüttmann and Lö nig [87].](image)

Optical imaging systems use light in the wavelength range between 400 nm and 900 nm. Compared to other diagnostic techniques like traditional X-ray CT scans, non-ionic radiation is used. Limitations of the optical imaging technique are mainly caused by the low penetration depth of light into body tissues [88; 89]. Visible (VIS) light like used for endoscopy is routinely used to examine tissue surfaces. The
imaging of deeper tissues (>500 μm) requires NIR light [88; 90; 91] which has much better penetration efficiency.

Hemoglobin, oxyhemoglobin and water are the major absorbers of visible light in biological tissues [92]. These three components have the lowest absorption coefficient between 650 nm and 900 nm (Figure 6).

![Absorption spectra of the 3 major biological light absorbers (water, hemoglobin, oxyhemoglobin). Within the NIR window the light absorption is minimal. Therewith, NIR light can be used for measurements in deeper body tissues. Figure adapted according to Weissleder et. al [90].](image_url)

This low absorption rate enables NIR fluorescence light to pass easily through body tissues. In the case of optical measurements the use of NIR light enables also to reduce the influence of disturbing background signals and consequently to achieve better signal to noise ratios. Therefore, this bandwidth is called the diagnostic- or NIR-window and is more and more utilized for *in vivo* fluorescence imaging.
C 1.2. Principles of fluorescence

Optical fluorescence strongly depends on the properties of the used fluorophore, the corresponding characteristics of excited and emitted light and on the surrounding properties (pH, polarity, etc). Three fundamental processes of the fluorophore can be considered after it is exited by a quantum of specific energy from the excitation light [93; 94]:

- Absorption of light energy, associated with an electron transfer to an excited state,
- Emission of radiation associated with relaxation and
- Non-radiative relaxation within and from the excited state.

A typical possibility to illustrate these electronic processes is the Jablonski diagram, shown in Figure 7.

![Jablonski diagram](image)

Figure 7: Jablonski diagram, illustrating energy processes. Figure adapted according to Lakowicz [95].

The singlet ground state \( S_0 \), in addition to the first \( S_1 \) and second \( S_2 \) excited singlet states are displayed in Figure 7 as a stack of horizontal lines. The thicker lines represent electronic energy levels, while the thinner ones denote the various vibrational energy states. The absorption of light occurs approximately in femtoseconds by what the fluorophore is usually excited from the ground state \( (S_0) \) to higher vibrational levels of the first \( (S_1) \) or second \( (S_2) \) singlet energy states.
The transferred energies (excitation and emission) are measured in terms of quanta and are expressed by the Planck’s Law:

\[ E = h \cdot \nu = \frac{h \cdot c}{\lambda} \]

where \( E \) is the energy, \( h \) is the Planck’s constant, \( \nu \) and \( \lambda \) are the frequency and the wavelength of the incoming photon respectively, and \( c \) is the speed of light. The Planck’s Law dictates that the radiation energy of an absorbed photon is directly proportional to the frequency and inversely proportional to the wavelength. The efficiency of the fluorescence process is normally specified by the fluorescence quantum yield, as the ratio of the emitted and absorbed number of photons. Immediately after absorption of a photon, several processes may occur with varying probabilities. The most likely is the relaxation to the lowest vibrational energy level of the first excited state \( (S_1 = 0) \), known as vibrational relaxation (loss of energy without light emission). Due to the loss of energy during this step, there is always a shift from higher to lower energies and the emission wavelength is bathochromic shifted. This phenomenon was first observed by the British scientist, George G. Stokes and was therefore termed Stokes’ shift \[95\].

If the relaxation from the lowest \( S_1 \) level is accompanied by an emission of a photon, the process is called fluorescence. This circular process of excitation and further emission can be repeated for most fluorophores many hundreds to thousands of times before the highly reactive excited state molecule is photobleached. The time, to the destruction of the fluorophore and the loss of further fluorescence, is one quality parameter of a fluorescence dye. In the case of fluorescein isothiocyanate (FITC) the molecule can undergo approximately 35,000 excitation and relaxation cycles before the molecule no longer responds to incident illumination \[96\].

**C 1.3. Dyes applicable for fluorescence imaging**

The ability of different molecules as well as of few proteins to emit photons, after their excitation with light requires conjugated systems and delocalized electrons. Un-flexible systems are preferred, due to constrained relaxation and thus increased fluorescence \[97\]. Diversified targeted \[98-100\], non-targeted \[101\] as well as of activatable fluorochromes \[99; 102; 103\], fluorescent proteins \[104-106\] and bioluminescent probes \[107; 108\] are commercially available. Depending on the chemical structure, those dyes can be excited with light of specific wavelengths (within VIS or NIR bandwidths). They have different properties and therewith specific capabilities.
Photobleaching should be considered as one characteristic property, especially when dyes are excited with high energy lasers as it can be the case in confocal microscopy studies. Dyes used for *in vivo* fluorescence imaging should have a combination of various other properties like high fluorescence quantum yields in the desired wavelength spectrum, sharp and characteristic emission spectra, sufficient biological stability to permit unimpaired image acquisition, and solubility in the respective environment. Also the chemical stability, the dye/protein binding ratio *in vivo* and specific targeting abilities have to be considered. Among numerous of organic fluorophores there are some often used particular dye classes like the polymethine dyes (e.g. cyanines, hemicyanines and benzopyrillium dyes), xanthene dyes (e.g. fluoresceins and rhodamines), oxazine dyes (nile blue) and oxazone derivates like nile red (NR) [109-111].

Altogether there are hundreds of fluorescent dyes available on the market. Also dyes with several reactive groups for direct protein or polymer labeling can be purchased. Most of the fluorescence dyes were synthesized to be used for fluorescence microscopy especially to visualize for example cellular structures and cell components. Dyes which are suitable for the *in vivo* characterization of parenteral formulations must emit fluorescence light at least above 500 nm. Light which is emitted at lower emission wavelengths would be absorbed by the body tissue nearly completely and could not or only hardly be detected by non-invasive optical imaging. A small, representative selection of dyes which is highly interesting for fluorescence imaging within this research topics is described briefly in the following.

Cyanine dyes are capable to cover the whole light spectrum from visible to far NIR wavelengths. The spectral properties of cyanines (e.g. Alexa Fluor dyes, Cy5, Cy7) and especially of dialkylcarbocyananine dyes (e.g. DiI, DiD, DiR) are mainly independent from the lengths of the alkyl chains. The fluorescence emission of the dialkylcarbocyananine dyes is mainly influenced by the heteroatoms in the terminal ring systems and the length of the connecting bridges (Figure 8). They are characterized to have very high extinction coefficients but rather moderate fluorescence quantum yields (30 % compared to xanthen dyes) and comparable short excited state lifetimes in lipid environments [110; 112]. Dialkylcarbocyananine dyes are nearly insoluble in water (log P values between 17.4 [113; 114] and 20 [115]) and they are weakly fluorescent in aqueous surroundings [116]. Compared to other lipophilic dyes like Cy5 the photobleaching rate of dialkylcarbocyananine dyes after 100 h excitation (<2 % in methanol solutions and 10 % when loaded to lipid carriers) is highly reduced and attests them ideally properties for *in vivo* applications
For *in vivo* imaging often used dialkylcarbocyanine dyes with corresponding fluorescence emission wavelengths are (according to the synonym of the distributor invitrogen): DiI: emission maximum (Emax) at 565 nm, DiD Emax at 665 nm and DiR with an Emax at 780 nm.

Compared to cyanine dyes, xanthene stains like fluorescein and rhodamine (Figure 9) are not available with fluorescence emissions far beyond 700 nm [110]. However, they exhibit extremely high quantum yields and are very good water soluble. Corresponding log P values are given to be between -2.4 and 2.8 [114; 115; 118]. Due to the hydrophilic properties, next to fluorescence emissions at around 540 nm, rhodamine can easily be used to stain hydrophilic drug carrier systems like gels or implants which allow making predictions of future hydrophilic drug releases [119; 120].

Fluorescein and many of its derivatives (the second xanthene dye family) have a pH-dependent fluorescence. Both, the phenol and the carboxylic acid, functional groups of fluorescein are almost totally ionized in aqueous solutions above pH 9. An
acidification of the di-anion, protonates the phenol (pKa about 6.4) first, yielding in the mono-anion. Both are fluorescent and can be used for pH dependent fluorescence measurements. However, the fluorescence emission spectrum is dominated by the dianion by what the wavelengths and the shapes of the emission spectra are relatively pH independent. Only the fluorescence intensity is reduced at acidic pH values. Based on fluorescein derivates were synthesized by Molecular Probes (now invitrogen): SNARF-1 (pKa ≈ 7.5) and SNARF-4F (pKa ≈ 6.4), shown in Figure 10 [121; 122].

![Figure 10: Chemical structures of 5-(and-6)-carboxy SNARF-1 (a) and SNARF-4F 5-(and-6)-carboxylic acid (b).](image)

These seminaphtho dyes are structurally not equivalent and exhibit dissimilar spectral properties [111]. The emission spectrum undergoes pH-dependent wavelength shifts. This allows the analysis of two emission maxima: typically at about 580 nm and at 640 nm and permits intensity independent pH detections at least in vitro for SNARF-4F between pH 5 and 7 [77; 79; 123].

Nile red (NR), an oxazone derivate is another often used dye (Figure 11). The log P value of NR is reported to be between rhodamine and that of cyanine dyes (3.8 [124] - 5.1 [125]). NR is a solvatochromic dye. The emission spectra vary in shape, position, and intensity with the polarity of the surrounding solvent [126]. The emitted light is shifted to shorter wavelengths with decreasing solvent polarities and the fluorescence is quenched nearly completely in aqueous media [127].

![Figure 11: Chemical structure of nile red.](image)
C 1.4. **Intrinsic limitations of in vivo optical imaging**

The multitude of fluorescence dyes with a large variety of properties and the ability to measure different native parameters like absorption, scattering, emission or spectral characteristics [128] might suggest an easy and wide use of fluorescence in vivo imaging. However, there are several intrinsic limitations when using fluorescence imaging. The spectrum of light which is passing tissues often varies by biochemical events. Emitted photons, detected by the imager, undergo multiple scattering events on their way through the body tissues [91; 128; 129]. Next to hemoglobin, oxyhemoglobin and water as the major absorbers, other tissue components can absorb light too such as:

- Small molecules like sugars, fatty acids, amino acids and nucleotides;
- Macromolecules like proteins, phospholipids, ribonucleic acid (RNA) deoxyribonucleic acid (DNA) and polysaccharides;
- As well as structures like organelles and cell membranes.

The general absorption occurs especially in the VIS wavelength range and is at least one order of magnitude less pronounced in the above discussed NIR window. Pelt as well as organs, which are highly supplied with blood like liver, spleen and kidneys absorb fluorescence excitation and emission light below 650 nm nearly completely. In the VIS wavelength range both, the limited ability of excitation light to penetrate into the skin (Figure 12, green spot) as well as the limited capability of light emitted from a fluorophore to pass the skin surface (Figure 12, yellow spot) hamper the in vivo use of fluorescence dyes emitting in the VIS range.

![Figure 12: Schematic illustration of excitation and emission processes using visible (green and yellow) and NIR (dark red) light for deep fluorescence imaging. Figure adapted according to Shuhendler [91].](image-url)
Due to the reduced absorption in the NIR range, NIR dyes are capable of deep tissue penetration. Multiple elastic scattering effects of photons are the main mechanism of light propagation of NIR light in body tissues [130]. By this phenomenon, NIR light transport within tissue can be modeled as a simple isotropic diffusing process [131]. Therefore, an exact localization of the emitting fluorescence light source beyond the surface becomes challenging. Depending on the depth of the fluorescent targets, structures obtained in the measurement images getting more and more blurred. Another consequence of this light scattering is an alteration of the intrinsic fluorescence emission spectra during the way through body tissues [128; 132]. This effect depends on optical properties of the tissue, the depth of the fluorophore, and is additionally influenced by the geometry and the wavelength of the light irradiation. In consequence the shape and the peak position of the detected fluorescence spectra may vary depending on the structure and the path lengths through the tissue [92; 133; 134]. Emission spectra of NIR dyes are very smooth and have no detail information like characteristic peaks. Also spectral shifts are not as good detectable as it is possible for fluorophores, emitting in the VIS band width. Dyes with emissions below 650 nm have higher quantum yields and more characteristic spectral shapes. Variations in the emission peaks are more easily detectable.

**C 1.5. Challenges when using *in vivo* imaging**

The auto-fluorescence of various other molecules in the animal feed and in different body tissues is next to the absorption and scattering effects another main limiting factor. Collagen, elastin, flavins, NADH, porphyrins, pyridoxine, tryptophan as well as chlorophyll, a component of animal fodder for instance are well known as auto-fluorescence molecules [135; 136]. These natural fluorophores emit light usually at lower VIS wavelengths. Their influence on far-red and NIR fluorescence imaging is often negligible. To circumvent that fodder influences measurements, special non-fluorescent chow can be used. Auto-fluorescence resulting from the body tissues is mainly initiated by the pelt and the skin of the mice. This may substantially overlay emitted fluorescence light. To sensitively track dyes *in vivo*, hair above the measurement regions should be removed completely. This can be performed only for smaller skin areas, whereas shaving is inefficient and depilation with foam or cream requires additional narcosis, results in skin irritation, and increases the risk of cooling out. However, also for small regions these strategies are insufficient for long-term measurements as the hair grow
fast. Therefore, the use of nude, hairless mice like SKH1-Hrhr or NU-Foxn1nu is preferred for comprehensive fluorescence in vivo measurements.

All factors mentioned above hamper the in vivo imaging. To increase the selectivity and the sensitivity of detecting fluorescence dyes in vivo, additional imaging and analyzing techniques were commercially developed. Some in vivo fluorescence imaging systems reduce auto-fluorescence influences by special spectral un-mixing techniques. This enables to perform certain corrections of the undesired auto-fluorescence signals [137]. Other measurement factors like imperfect filtering, different light sources as well as of different analyzing software influence the outcome of in vivo measurements too. Even though all fluorescence in vivo imaging systems are rapid, painless and harmless to the animals, they are distinguished by the differences in sensitivity, handling, evaluation possibilities as well as by the functionalities in removing auto-fluorescence and scattering artifacts.

**C 1.6. Measurement systems for optical in vivo imaging**

The development of small animal imaging systems has progressed rapidly over the last 10 years. A wide variety of approaches and components using fluorescent or bioluminescent signals were developed [18; 138-140]. Bioluminescent systems have certain restrictions and are generally used for luciferase genes and with luciferin substrates as reporters [141]. These systems are promising but limited to special areas of application and therefore will not be further discussed in the current work.

Fluorescence based imaging systems require an external light source to excite the fluorophores. In the last 5 years, the Maestro imaging system and more than 5 other pre-clinical imaging systems were launched to the market [23]. This emphasizes the wide range of approaches and capabilities. All these systems are either reflectance mode based (light source and detectors are located on the same side of the tissue) or transmission mode based, which is constructed like a transmittance light microscope [130].

Another main differentiating characteristic of these imagers is the light source itself. Broad beam imagers are based on xenon, tungsten or halogen lamps. Other source systems are either LED- or laser-based [23]. Systems with broad beam light sources are simple, relatively inexpensive, provide rapid acquisition and can be applied for various dyes. The excitation wavelength for each respective dye can normally be adjusted using special light filters. Broad beam systems are generally configured in the reflectance mode. However, this increases the light scattering effects. Fluorescence emissions of fluorophores are overlaid by non-specific auto-
fluorescence signals. This requires the effective filtering of excitation as well as of emission light. But those systems allow measuring a large variety of VIS and NIR dyes.

In the beginning of this work, the newly launched fluorescence imager: Maestro \textit{in vivo} fluorescence imaging system form Cambridge Research & Instrumentation (Cri), United States (now PerkinElmer (Caliper Life Sciences), United States) was chosen to evaluate the possibilities of fluorescence imaging. The Maestro is a reflectance mode system equipped with a broad beam xenon lamp.

\textbf{C 2. The Maestro \textit{in vivo} imaging system}

\textbf{C 2.1. Instrumentation}

The instrumentation design of the Maestro \textit{in vivo} fluorescence imaging system is shown in Figure 13. The Maestro system is equipped with a 300 Watt Cermax-type, xenon lamp. The lamp emits light in the range between 500 nm and 950 nm. Undesired light can be blocked by one out of six available excitation filter (Figure 13 a, left).

![Figure 13: Schematic illustration (a) of the main Maestro \textit{in vivo} imaging components and a photograph (b) of the imaging unit of the Maestro \textit{in vivo} imaging chamber.](image)

The excitation filter specifications for all available Maestro filter sets are given in Table 1. As specified in the table the excitation filters have comparable narrow pass widths. Light beyond the limits is blocked completely. Only light of a certain wavelength range can pass the respective filter and excite the fluorophore. This reduces the amount of auto-fluorescence.
Table 1: Available Maestro filter sets.

<table>
<thead>
<tr>
<th>Filter set</th>
<th>Excitation filter</th>
<th>Emission filter</th>
<th>Standard acquisition setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>445 to 490 nm</td>
<td>515 nm</td>
<td>500 to 720 nm, in 10 nm steps</td>
</tr>
<tr>
<td>Green</td>
<td>503 to 555 nm</td>
<td>580 nm</td>
<td>550 to 800 nm, in 10 nm steps</td>
</tr>
<tr>
<td>Yellow</td>
<td>575 to 605 nm</td>
<td>645 nm</td>
<td>630 to 850 nm, in 10 nm steps</td>
</tr>
<tr>
<td>Red</td>
<td>615 to 665 nm</td>
<td>700 nm</td>
<td>680 to 950 nm, in 10 nm steps</td>
</tr>
<tr>
<td>Deep red</td>
<td>671 to 705 nm</td>
<td>750 nm</td>
<td>730 to 950 nm, in 10 nm steps</td>
</tr>
<tr>
<td>NIR</td>
<td>710 to 760 nm</td>
<td>800 nm</td>
<td>780 to 950 nm, in 10 nm steps</td>
</tr>
</tbody>
</table>

Fiber optics and height adjustable arms ensure that the excitation light (Figure 13 a, blue line) illuminates the object homogeneously. Emitted fluorescence light (Figure 13 a, red dotted line) passes an appropriate long pass emission filter (specified in Table 1) which blocks the excitation light completely. The blocking of light with lower wavelengths by the long pass emission filter minimizes also the amount of auto-fluorescence light that can interfere with the desired specific fluorescence signal. Afterwards, the emission light passes an objective which includes a downstream tunable solid-state liquid crystal element filter (Figure 13 a, top, specified in Table 1). These liquid crystal element allow to control that only specific wavelengths in predefined intervals can pass the objective (10 nm intervals are by default predefined). By this, only desired wavelengths are detected by the scientific grade charged-coupled device (CCD) light sensor which acquires the measurement images.

C 2.2. Measurement fundamentals

Measurements using the Maestro in vivo imaging system are performed in three steps:

1. Acquisition of multispectral fluorescence images
2. Spectral unmixing and auto-fluorescence removal
3. Analyses of measurement files

These three main imaging steps are common for all in vitro, ex vivo and in vivo experiments. An in vitro pH measurement was used as an example to discuss the three steps briefly in the following.

This experiment was one of the prerequisites for the in vivo pH detection of in situ forming implants (see section G Long-term in vivo pH measurements of in situ forming PLGA implants). Within the experiment, three Eppendorf cups filled with
aqueous solutions of different pH values (pH 5, pH 6 and pH 7) were dyed with SNARF-4F. As described above the emission spectrum of this dye undergoes a pH-dependent wave length shift which can be afterwards used for unmixing and analyzing tests. Within the experiment, all three Eppendorf cups were imaged simultaneously.

C 2.3. Acquisition of multispectral fluorescence images

The multispectral analyses are based on the principle that all fluorescent molecules have unique emission spectra. If a fluorescent sample is excited, the emitted fluorescence is distributed over a range of wavelengths of varying emission intensities. To ensure the detection of the unique wavelengths, the Maestro software controls a tunable liquid crystal filter and the CCD chip. This allows the automatic acquisition of a set of multispectral images at predefined wavelength intervals. Figure 14 depicts the resulting images. The pictures were acquired with the green filter set between 550 nm and 800 nm and additionally with the yellow filter set between 700 nm and 850 nm using a predefined wavelength interval of 50 nm. All other measurements within this research work were performed with intervals of 2 nm or 10 nm. The start and the end wavelengths were defined in accordance to the respective filter set and the corresponding acquisition settings (compare Table 1). An acquisition in 2 nm steps increased the available spectral information while extending the measurement time. A recording in 10 nm steps accelerated the measurement, which minimizes motion in the in vivo records.

![Figure 14: Two grayscale image sets (50 nm steps, top: green filter set, bottom: yellow filter set) of a sample pH measurement (3 Eppendorf cups, pH 5, 6 and 7, dyed with SNARF-4F).](image-url)
Chapter I: Introduction - Basic principles

The CCD chip records one 12 bit file (intensity values between 0 and 4,096) at each wavelength interval. Images of the files captured in 50 nm intervals are exemplarily shown in Figure 14. As the emission intensity and consequently the signal to noise ratio is normally very low at the beginning and at the end of a measurement set (see also Figure 16), the overall signal intensity for the visualization is amplified to display slight intensity variations. Images are grey with high noise levels. Images at the maximum emission wavelengths have a very high signal to noise ratio. The total signal is not amplified and the images have an excellent contrast ratio. Thus the background is displayed black and the measured emission graduated white. For all pixels in the images there is a corresponding intensity value which is used for the unmixing calculations.

Altogether, each pixel displayed in the image is related to the measured intensity value of the probe at this wavelength.

Intensity values of all images, captured at each defined wavelength interval are stapled to one working file: ‘cube’ (Figure 15). The cube is displayed as a red-green-blue (RGB) color image. The colors are related to the respective acquisition wavelengths. This facilitates the manual handling in selecting for example a defined region of interest (ROI) without influencing the software analysis.

![Figure 15: Image of a Maestro working file: ‘cube’. Within this cube all recorded images shown in Figure 14 are stapled. The file is automatically RGB colored which is helpful for the analyzing process.](image)

Each single pixel in the cube file contains the intensity information of all images stapled in the cube file. The more images were made, the more information is available for each pixel of the cube. Based on the intensity values of all single images, the emission spectrum can be extracted for each pixel or group of pixels of the cube. This spectrum varies more or less from pixel to pixel and is specific for each respective fluorescent sample.
C 2.4. Spectral unmixing and auto-fluorescence removal

A fluorescence emission spectrum can be extracted manually or automatically by combining the spectral information of a certain group of pixels. This emission spectrum is composed of at least two single spectra: the emission spectra from the dye itself and the background signal. In the case of the imaged Eppendorf cups in Figure 15, the spectrum is composed of:

- The SNARF-4F emission spectra (varying depending on the respective pH value in the Eppendorf cup) and
- The potential signal of the plastic material of the Eppendorf cup itself.

However, also the overlapping of two or more emission spectra can occur. This is for example the case if different dyes are imaged simultaneously or if the spectral shift of a dye due to diversified surrounding properties occurs. Also the spectral change caused by in vivo interactions could result in varying spectra.

For further detailed analysis the isolated dye spectrum loaded into a spectral library is needed. Disturbing background signals must be recomputed. Therefore, the Maestro in vivo imaging system uses patented algorithms. Those, allow auto-fluorescence removal, spectral unmixing and fluorescence quantification analyses.

Generating the spectral library is a crucial step within the spectral unmixing process. The spectral library is the basis for the analysis of all cubes within a series of measurements. Spectral libraries within this research work were mostly be generated manually. Therefore, control samples measured under same conditions as the probes itself were used. Many main prerequisites had to be clarified especially for in vivo experiments such as:

- Is the emission spectrum changed by surrounding properties or overlaid by other spectra?
- Are in vitro and in vivo spectra comparable?
- Does the spectrum depend on the measurement depth?
- Do different body tissues have differentiating auto-fluorescence signals?
- Is the auto-fluorescence depending on the measurement depth of the fluorophore in the body?
- Is the background signal depending on the wavelength of the excitation light?
- Is the spectrum itself and/or the ratio between the dye and the background spectrum exposure time dependent?
For advanced spectral calculations using other software, generated spectral libraries can also be exported. Figure 16 depicts the isolated spectra of the sample cube shown in Figure 15.

**Figure 16:** Intensity weighted (a) and to the maximum, normalized intensity (b) graphs of the isolated SNARF-4F emission spectra at pH 5 and pH 7 of the left and right Eppendorf cup shown in Figure 15. The background signal was extracted measuring empty plastic Eppendorf cups under same conditions. All measurements were performed using two filter sets (green, yellow).

The generated spectra can be used to analyze the cube file. This spectral unmixing step is based on mathematical disentanglement of the measured spectra. The Maestro software calculates for each pixel in the cube if the pixel is either assignable to the background signal, to the spectral species of the dye or proportionately to both of them. The software has to consider that signals might variably be mixed also with
different, unknown amounts of dyes and auto-fluorescence [142]. Thereby, the software estimates the spectral shape of the putative, pure component based on spectral differences between the background and the dye spectra. By the help of the software the signals can be differentiated and quantitatively unmixed. Even very similar spectra with varying peak emissions of no more than 2 nm to 3 nm can be unmixed reproducibly [137; 143]. In the unmixing analysis, each pixel of the cube can be fully, partly or not assigned to a single spectrum of the used respective reference library. Using this data, respective intensity weighted grayscale images are generated for each spectrum of the used spectral library (Figure 17).

**Figure 17:** Unmixed grayscale images of the cube (Figure 15). Each picture summarizes the pixel information which was assigned to the extracted reference spectra (shown in Figure 16) of pH 5 (a), pH 7 (b) and the background (c).

### C 2.5. Analyses of measurement files

Various strategies can be applied for further analyses of the obtained, unmixed grayscale images. The Maestro software enables an advanced image processing for the visualization of the fluorescence allocation as well as of data analysis to determine values like the threshold signal areas [144]. The evaluation of the best and most meaningful analyzing method either image processing and/or data analysis has to be identified in numerous preliminary experiments.

The image processing is based on the obtained individual component grayscale images which are calculated after the unmixing step (Figure 17). Based on the grayscale distribution in the files an incremental jet color profile can be used to generate intensity-weighted images (Figure 18 a and b).

Pixels with maximum intensities are set to dark red and pixels with zero fluorescence to black. In between there is a gradation from red to orange, yellow and then to light
and to dark blue. This allows the visualization of intensity distributions in the respective extracted signal images.

The same principle can be used for a set of chronological sequences of images analyzed with the same reference spectra named as ‘compare imaging’ function. This enables to analyze multiple grayscale images within a measurement series related to their individual measurement conditions. Time-dependent variations can be displayed independently from varying measurement conditions like different exposure times. Also RGB composite images can be generated based on the unmixed grayscale images. Those RGB images (unmixed composite images) enhance the visual expressiveness of the unmixed grayscale images. Conclusions about the formation of separated signals in the cube can be made. Unmixed RGB pictures are generated while allocating respective manually chosen colors to the grayscale spectrum. In Figure 18 c these principles were used exemplarily. Green was assigned to pixels allocated to the pH 5 spectra, red to the pH 7 emission spectra and black to the unmixed background signal.

![Image of pH 5, pH 7, and unmixed composite images](image)

Figure 18: Intensity weighted images (a and b) of the corresponding unmixed grayscale pictures. Unmixed composite image (c) generated in assigning green to pH 5, red to pH 7 and black to the background signal.

Image processing using algorithms of the Maestro software allow the visualization of imaging results. However, the underlying data is quantitative. Intensity information is available for each pixel in an unmixed grayscale image which was allocated to the respective reference spectrum.
Based on this underlying data several parameters can be calculated by the Maestro software for a defined region of interest (ROI):

- Average signal, as the average intensity value of all pixels in the ROI
- Total signal, as the sum of all the pixel intensity values in the ROI
- Total signal related to the exposure time
- Maximum signal, as the maximum pixel intensity value included in the ROI
- Maximum signal related to the exposure time
- Area as the number of pixels or the size of the ROI

For further calculations, these generated data can also be exported to spreadsheets and other analyzing programs like Microsoft Excel. Altogether the Maestro analyzing software provides a variety of functions which have to be evaluated for each project as well as to each series of measurements, in order to identify the analyzing method with the highest expressiveness for the respective research objective.
Chapter II: Results of pre-clinical in vivo studies of polymers

D  Long-term in vivo biodistribution studies of i.p. injected high molecular weight PVA


D 1. Summary

Long-term in vivo studies of i.p. administered high molecular weight (195 kDa) PVA were performed to investigate the possibilities and constraints of using the non-invasive fluorescence imaging technique for the characterization of the water soluble polymer PVA [1-3]. To examine the body distribution, accumulation, and elimination processes by means of fluorescence imaging, the polymer had to be labeled with fluorescence dyes. For this purpose, two different dyes, either TMR (a rhodamine dye) or Alexa Fluor 750 (a NIR dye) were covalently bound to the PVA backbone by the group of Prof. Dr. habil. Jörg Kreßler [1; 2]. Both covalent labels were stable in vivo and had different spectral and optical properties. Imaging the TMR and the NIR labeled PVA allowed thorough in vivo biodistribution studies over several months.
Chapter II: Results of pre-clinical in vivo studies of polymers

It was observed that after *i.p.* injection into nude mice, the labeled PVA was mainly distributed by passive diffusion processes which were accelerated by the motion of the gastrointestinal tract and also after the PVA uptake into the bloodstream. An imaging of the blood vessels after 24 h confirmed the long-term circulation ability of the water soluble polymer in the bloodstream [3].

Non-invasive fluorescence imaging experiments indicated that PVA is highly accumulated in the different body fat tissues such as abdominal fat, kidney fat and also in fat layers under the skin [1]. Analyzing small spectral variances between the fluorescence emissions of labeled PVA from different body fat tissues, multispectral fluorescence imaging allowed for the first time even to differentiate non-invasively between the accumulations of PVA in different fat tissues. In addition, the PVA accumulations in the fat depots of male and also female mice were quantified over more than 6 months. This enabled calculation of the different release rates for both genders. The respective analysis indicated to a continuous, long-term release of PVA. *Ex vivo* fluorescence imaging studies of both labeled PVA polymer batches and additionally performed confocal microscopy studies confirmed the observed enrichment effect. It has been found that PVA molecules are accumulated in high concentrations between fat cells. Fluorescence imaging of the *in vivo* stable NIR labeled PVA polymer enabled further studies of PVA accumulation, its release and elimination pathways also in deeper body tissues as well as in organs which are highly supplied with blood [1]. High intensity fluorescence signals were detectable in the kidneys for up to 3 days *in vivo*. This confirmed urinary excretion studies which were performed with TMR labeled PVA [2]. Urine samples were collected and *in vitro* imaged by the Maestro imaging system. The amount of excreted PVA decreased over 5 days until the concentration fell below the detection limit of the imager [2]. This confirmed previous PVA studies in rabbits [34]. A slight accumulation of high-molar mass PVA was also detected in the liver in *ex vivo* experiments. This effect could be attributed to the high level of blood supply to the liver causing the preferential transport and accumulation of labeled PVA. Finally, PVA was eliminated from the liver by feces. Nine months after *i.p.* application, the PVA-NIR fluorescence in the liver was below the detection limit. Therewith, a complete elimination of PVA from the organism can be expected [1]. Based on the studies performed the accumulation of PVA molecules in various fat tissues, observed *in vivo* and confirmed *ex vivo*, seems to be non-critical for clinical PVA use. No evidence indicating that PVA could accumulate permanently in body tissues or that it is toxic to cells was found within these studies. The results regarding the *in vivo* release of PVA from fat tissues
Chapter II: Results of pre-clinical in vivo studies of polymers

attested PVA to be potentially suitable for a controlled long-term release of bound drug molecules or proteins. Within these studies it has been shown that fluorescence imaging is very helpful tool for the pre-clinical characterization of the biodistribution and excretion pathways of polymers.
Chapter III: Results of pre-clinical in vivo studies of nanoparticles

In vivo characterization of nanocarriers and their potential capability in cancer therapies


E 1. Summary

It is known that serious side effects occur very often due to nonspecific distributions of chemotherapeutic agents to both cancerous and normal cells. Often this is also the reason why the cancer therapies are terminated. To avoid this, drug carriers are needed to deliver their toxic cargo specific to cancer cells. The particle size distribution of the formulation is one of the key parameters for the nanoparticle accumulation in tumor tissues [59; 61; 62; 145].

To investigate the influence of particle sizes and to research the nanoparticle biodistribution, accumulation and elimination processes in detail, in vivo and ex vivo fluorescence imaging studies were combined with thorough particle size characterizations [4; 5]. Polyethylene glycol - polylactide acid (PEG-PLA) block copolymers were synthesized in the group of Prof. Dr. Achim Göpferich and were used for the production of defined, stable, and nontoxic NIR dye-loaded nanoparticle batches [52]. PEG with an average molecular weight of 2000 Da was covalently bound to PLA to prolong the estimated in vivo circulation time of the NPs [4]. Nanoparticles were lyophilized to assure long-term stability [52]. Performed particle size experiments indicated that even aqueous dispersions, stored at 5 °C were stable.
over at least 3 months. Cell toxicity experiments with PEG-PLA NPs showed that they are biocompatible and non-toxic to CHO and L929 cells [4; 52].

To investigate the size dependent in vivo fate of PEG-PLA nanoparticle, well-defined batches varying in size were needed. Therefore, asymmetrical field flow field fractionation (AF4) coupled with multiangle laser light scattering (MALLS), photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM) were used to determine the particle sizes of produced nanoparticle batches.

The results of AF4/MALLS showed that even in batches with rather low polydispersity indices (e.g. PCS z-average = 166 nm, PDI = 0.13), 10 % of the nanoparticles were smaller than 48 nm (D10) and the same amount of particles was larger than 230 nm (D90) [5]. This confirmed the need to combine different size measurement techniques for detailed particle size characterization as consumption for size-dependent biodistribution studies. For in vivo experiments several nanoparticle batches varying in size were produced and characterized [5]. The particle size z-averages of the batches (between 111 nm and 166 nm) are in the size rage which is reported to be best suitable for tumor targeting [55].

Initial in vivo fluorescence imaging experiments were performed with nile red (NR) loaded nanoparticles [4]. The results indicated the fast release of the NR dye from the particles and the rapid dye elimination from the bloodstream. The incorporation of the highly hydrophobic NIR dye DiR which is not released from the particles enabled the detection of circulating nanoparticles in the bloodstream for up to 6 h after injection [4]. This time period should be sufficient for the accumulation of nanoparticles in tumor tissues.

The in vivo fate of produced NIR nanoparticles was carried out in nude mice using the Maestro in vivo fluorescence imaging system [5]. The multispectral fluorescence imaging technique made it possible to follow the in vivo fate of the particles non-invasively even in deep tissues over several days. These experiments were combined with detailed fluorescence ex vivo imaging studies. The combination of in vivo and ex vivo imaging appeared to be a promising approach to study nanoparticle accumulation in different tissues and organs. Therefore, a new calculation approach was developed to calculate the total and maximum fluorescence intensities [4]. This enabled the comparison of nanoparticle batches with varying fluorescence intensities and provided the basis for the determination of the influence of varying particle sizes on the in vivo fate.

Based on these results tumor accumulation studies were performed in order to investigate the influence of particle sizes to their accumulation potential in tumor
Chapter III: Results of pre-clinical in vivo studies of nanoparticles

tissues [5]. For this purpose, two human xenograft tumor models with different shapes and growth rates: the colon carcinoma (HT29) and the ovarian carcinoma (A2780) were chosen.

Non-invasive multispectral fluorescence imaging using the Maestro in vivo imaging system enabled the simultaneous combination of the visualization of DsRed2 expressing HT29 xenografts and the in vivo tracking of NPs [5]. Ex vivo imaging together with additional confocal microscopy studies facilitated to reveal also information about the accumulation characteristics of the nanoparticles inside the tumor tissues. The confocal microscopic pictures confirmed the ex vivo imaging results. The particles could be detected between fluorescent, DsRed2 expressing cells [5]. In vivo studies with HT29 and A2780 tumor bearing mice indicated that nanoparticles accumulated in both tumor models. The ex vivo studies furthermore demonstrated that the accumulation pattern mainly differed between both researched tumor models. Nanoparticles enriched primarily in the tumor center in HT29 tumor tissues whereas A2780 showed no centralized nanoparticle accumulation [5].

To investigate the size dependent tumor accumulation ability in both tumor models the respective total and maximum fluorescence intensities over several hours were calculated for three NIR PEG-PLA nanoparticle batches [5]. Two nanoparticle batches (111 nm and 141 nm in diameter) accumulated efficiently in the human xenograft tumor tissues. The highest tumor enrichment was found for the nanoparticle batch with a z-average of 111 nm for the A2780 tumors. Whereas the 141 nm nanoparticles were most accumulated in HT29 tumors [5]. This emphasized the fact that slightly larger nanoparticles (z-average of 141 nm) seems to be beneficial for accumulations in necrotic (HT29) tissue, whereas a slightly smaller size (z-average of 111 nm) was preferable for vascular permeation. Light microscopic studies of thin section slides of tumor tissue confirmed the accumulation in necrotic tissues for HT29 xenografts. Bigger nanoparticles (z-average of 166 nm) were not effective in both xenografts [5]. It can be expected that they are eliminated rapidly by the liver and therewith the accumulation in the tumor tissues was ineffective.

Visualizing the nanoparticles and the dyed cells simultaneously in in vitro experiments with A2780 and HT29 cell layers using confocal microscopy indicated the ability of the particles to bind to tumor cells.

Based on these experiment results, the combination of extended accurate size determinations with in vivo fluorescence imaging technique appeared to be a very promising approach to study the size dependent fate and the tumor accumulation potency of nanoparticles.
Chapter III: Results of pre-clinical in vivo studies of nanoparticles

F Investigating the potential toxicity risk of nanocarriers


F 1. Summary

Many different nanocarrier formulations are already frequently used in modern pharmaceutical therapies or are intensively researched to be used in future for example in anticancer therapies [53; 60; 146]. However, compared to traditionally used drug dosage forms, the application of nano-scaled formulation systems may result in different in vivo behaviors and in new potential toxicity risks due to varied physicochemical properties.

Therefore, this research work was besides the characterization of the biodistribution and the tumor accumulation behavior also focused on unusual distribution and accumulation patterns of injected nanocarriers, indicating potential toxicity risks. Within the performed in vivo characterizations it was found that used PEG-PLA nanoparticles seems to accumulate in rodent ovaries after i.v. administration [6]. The further research was focus to investigate the identified accumulation in ovarian tissues in detail. The effect was confirmed in different mouse species and also in Wistar rats. Numerous additional in vivo fluorescence imaging studies were performed while using other nanocarrier systems (nanoparticles, nanocapsules and nanosized lipid emulsions) which were differently composed, including multiple excipients, carrier sizes and surfaces. An accumulation in specific compartments in the rodent ovaries was found for all original and additional researched nanocarriers [6].

All tested nanocarrier batches with diameters between 45 nm and 350 nm accumulated in high concentrations in the ovaries, whereas tested polymers were not accumulated [6]. A comparison of three nanoparticle batches varying in size led to the conclusion that bigger particles seemed to be more accumulated than smaller ones. The accumulation found in vivo was further characterized by ex vivo fluorescence imaging and confocal microscopy. Based on the size and structures of the accumulation areas identified in the performed experiments, enrichments in tertiary vesicular follicles are possible. However, the nanocarrier accumulation in
cells of the *corpus luteum* seems to be more likely. The *corpus luteum* is formed from the wall of the ruptured follicles after the ovulation. It is described that the rates of tissue growth and angiogenesis in the *corpus luteum* rival those of fast growing tumors [147]. This supports the assumption that the accumulation of the nanocarriers in the *corpus luteum* is related to the enhanced permeability and retention effect known for tumor tissues. By this, nanocarriers have the ability to be retained in tumor tissues which is a highly size dependent effect.

Altogether, this is the first study which comprehensively investigated the accumulation of nanocarriers in the ovaries. Reasons, why the nanocarrier accumulation had not yet attracted attention might be the fact that often male mice or not yet pubescent female mice are used. In addition, the nanocarrier accumulation appears to be restricted only to certain local parts of the ovaries. Thus the overall amount of fluorescence signal from the total ovary tissue was rather small and comparable to other organs and tissues where no nanoparticles accumulated [6]. Usually, in *in vivo* studies the average nanocarrier amounts are related to the organ/tissue weight or to the administered dosages. This information is then used to compare different body compartments. Therefore, the accumulation of nanocarriers in the ovaries might remain unnoticed although it can be concluded based on a small number of other published results which refer to *in vivo* and *ex vivo* studies with different kinds of nanoparticles and liposomes [148-154]. Newly published studies identified also accumulations and/or potential toxicological effects of silver and of titanium dioxide nanoparticles as well as of nanocapsules in rodent ovaries [155-157]. These results confirm the found accumulation of nanocarriers.

It has to be noted that the accumulation of the nanocarriers in the ovaries does not necessarily need to result in a risk for the widespread use of nano-scaled carrier systems in medicine. Especially due to the fact that the accumulation is limited to special regions in the ovaries, the toxic risk for humans might be rather low. However, this effect should be further investigated, particularly in other species and with other nanocarriers, to elucidate the mechanism of the accumulation. Also the chances of these results for a new ovarian targeted therapy should be taken into consideration.

The results strongly emphasize the relevance of early explorative *in vivo* studies in the development of drug delivery systems using sensitive analytical imaging techniques like fluorescence imaging using NIR fluorescence dyes.
Chapter IV: Results of pre-clinical in vivo studies of in situ forming implants

G Long-term in vivo pH measurements of in situ forming PLGA implants


G 1. Summary

In the last decade the number of peptides and proteins used for medicinal therapies increased continuously. However, peptide and protein delivery has been a challenge due to their limited stability during preparation, storage and in vivo release. They have to be parenteral administered e.g. via intramuscular (i.m.) or s.c. routes.

Several parenteral drug delivery systems like microspheres, solid implants and in situ forming implants were developed for peptide delivery to achieve constant therapy levels and to reduce the application frequency [67; 158]. These formulation systems enable a controlled release of incorporated therapeutic agents over several days, weeks up to months and improve the therapy compliance of the patients. Furthermore, these depot formulations minimize the fluctuation of drug plasma levels and therewith undesirable side effects. In situ forming implants can be injected with syringes into the target site, where the implant is formed immediately. PLGA is the most widespread polymer used for implants. One of the major concerns using PLGA is its uncontrolled pH drop in those controlled release drug delivery systems which can causing the instability of encapsulated drugs or proteins [76; 77; 159; 160]. Therefore, it is important to measure the microenvironment in PLGA implants and to understand the relationship between pH shift and the implant degradation.

In vitro EPR experiments showed that during incubation under ‘physiologic’ conditions the pH value can drop from pH 7.4 to values around pH 3 [7]. Due to fluctuating fluorescence intensities in in vivo surroundings as well as of the disturbing
auto-fluorescence of mouse tissues dyes with two (pH dependent) emission maxima at longer preferably wavelengths were needed. Only one dye, SNARF-4F (pKa of about 6.4) fulfilled those defined measurement requirements.

Thorough in vitro and ex vivo fluorescence imaging experiments were performed using the SNARF-4F dye to evaluate the influence of factors like auto-fluorescence of the skin, light absorption, intensity variations, measurement conditions and others. Those tests enabled the development of a method that allows calculating pH values independently from the fluorescence intensity. Therefore, a Boltzmann plot based on more than 40 ex vivo measured standard buffer solutions was computed [7]. Additionally visual evaluation of the captured fluorescence imaging measurement files was performed and tested in vitro.

All these tests were the basis to non-invasively measure and visualize the microclimate pH (μpH) value of PLGA implants in complex in vivo surroundings. In addition, the physically entrapping of the hydrophilic SNARF-4F enabled to measure the release profiles of the dye from the implant. It could be shown that the solvent/non-solvent exchange after injecting the PLGA/PEG 400 solution is very fast. After 1 h about 70% of the PEG 400 was replaced by body fluids and the implant started to solidify. During the next hours body fluids and remaining PEG 400 were eliminated from the implant. After three days the emission intensity in the implant was smallest as the implant was completely solidified [7].

The μpH measurements revealed that within the first 2 - 3 days the μpH remains constant. Afterwards, the μpH decreased continuously to the lower pH detection limit of pH 5.0. This was found in vivo and ex vivo. The results are in good accordance with the current knowledge of the implant degradation processes [161]. The subsequent pH increase as well as the continuous dye release from the implant within this phase was measured additionally and visualized in detail. However, the pH drop caused by the PLGA degradation products was observed to be slower in vivo.

The results confirm some in vitro and in vivo EPR experiments and are in accordance with published in vitro confocal laser scanning microscopy measurements [78; 79; 159; 162-164]. The experiments showed that in vitro pH measurement results are not by implication transferrable for the estimation of in vivo behaviors. The obtained results underlined that the evaluated fluorescence imaging method is a non-invasive method which allows the mapping of μpH values in vivo.
Chapter V: Results of alternative application fields of fluorescence imaging

Monitoring internal pH gradients in swelling multi-layer tablets


H 1. Summary

The techniques and principles in measuring pH values of in situ forming PLGA implants in vivo were as well applied for in vitro pH measurements in swelling multi-layer tablets. Varying pH values in different section of the human intestine highly influence the release and the uptake of especially ionizable drugs from tablets. The solubility of those drugs highly depends on the surrounding pH value. APIs which are not dissolved completely in vivo are normally not able to enter the blood circulation. Thus influencing the microclimate pH (μpH) within solid formulations is used to achieve pH-independent API release profiles. Fluorescence imaging was used to measure μpH values non-destructive in swelling two-layer tablets [8]. The measured μpH gradients were compared with two other pH measurement techniques: incorporating pH indicator dye and cutting the tablets after different time points as well as with EPR imaging. The obtained results of all three methods were comparable. However the informative value and the research findings were different. Even though the developed principles of calculating pH values using fluorescence imaging were applied, comprehensive pretests had to be performed additionally to evaluate issues like the influence of used excipients, best suitable pH sensitive dye and the finally needed dye concentration. To get a consistent measurement surface area and to limit the hydration process to one-dimension, the tablets were placed into transparent tubes with the exact size of the tablets. Thus the swelling of the tablets was only possible from the top and the bottom of the tablets [8]. This setup emphasized as the best method to measure the
μpH variation under the current experimental conditions. By means of fluorescence imaging the spatial μpH distribution of the tablet surface could be non-destructively obtained across the swelling process. However inner regions of the tablet were accessible only by cutting the tablet.

The fluorescence imaging results demonstrated that the incorporation of internal buffer substances as well as the nature of used matrix forming excipients strongly influences the μpH.
I Monitoring temperature distributions in tablets - caused by the tableting process

1. Summary

It is generally known that changes in temperature can have crucial effects on APIs. The processing and the storage of APIs below or above defined temperatures can cause chemical modification and degradation of the API. It is also known that increased temperatures in different parts of the tablet during the tableting process can lead to polymorph changes of the incorporated API [165]. Different temperature ranges in the tablet can be expected during the tableting process due to the heterogeneity of the pressure distribution [165].

Until now it is unclear if this is caused e.g. by the mechanical pressure, the choice and the humidity of the used excipients or the porosity of the tablet. Therefore, an in situ measurement system is needed which would enable to measure the temperature distribution during the tableting process.

To perform temperature burden measurements using fluorescence imaging a temperature sensitive dye had to be found which changes the fluorescence emission spectra at higher temperatures irreversible. In addition the dye had to be very sensitive so that it can be added in low concentrations to prevent interactions with other excipients. In addition the dye had to change the emission spectra independently from pressure but immediately to changes in temperatures. Permatherm Concentrate Magenta 60° was identified to fulfill all identified requirements [165]. The dye has an irreversible color change between 40 °C and 80 °C. The existence of two emission maxima enabled to calculate the temperature independently from concentration and intensity changes.

Temperature calibration experiments were performed based on comparable principals which were emerged for the in vivo and in situ pH measurements [165]. Due to limitations of the Maestro software, emission spectra of desired ROI were exported and analyzed manually. The temperature which occurred during the tableting was fivefold determined for six mixtures and for three tableting processes [165]. The results demonstrated that temperature maxima varied depending mostly on used excipients and partly on the applied tableting conditions. It could be demonstrated that not only the used excipients influence the maximum temperatures. Also the specific humidity of the used excipients affected the temperature burden.
Chapter V: Results of alternative application fields of fluorescence imaging

**J In vivo and ex vivo characterization of nanocapsules**

(IX) Li, J., Schädlich, A., Hause, G., Vogel, J., Kuntsche, J., Groth, T., Mäder, K., Pre-clinical in vivo studies of oily core PEG-PLGA Nanocapsules using fluorescence imaging. (in preparation)

**J 1. Summary**

Compared to other colloidal carriers nanocapsules have the advantage to be able to incorporate comparable high API amounts. The nanocapsules are composed of a liquid core, which can be loaded with API and a solid shell. The distribution of the nanocapsules in vivo as well as their degradation is triggered by the varying composition and the thickness of the shell. It has been reported that hydrophilic polymers (e.g. PEG) on the surface can reduce the opsonisation and thus the uptake by the mononuclear phagocytic system (MPS) [4]. However there is a lack of data available concerning the in vivo distribution of nanocapsules and the effect of PEG on the surface of the nanocapsules. Therefore, two very lipophilic fluorescent dyes either DiI or DiR were incorporated into the oily core to characterize the in vivo fate of oily nanocapsules with different composed shells by means of fluorescence imaging [166]. This was performed using methods which were established for the in vivo and ex vivo characterization of nanoparticles [4; 5]. Comparable particle sizes (z-average around 150 nm) were measured for all tested nanocapsule batches [166]. In vivo fluorescence imaging results showed that these nanocapsules circulated in the bloodstream for several hours. This confirmed the effectiveness of the PEG surface. The highest nanocapsule concentration was determined in the MPS 24 h after i.v. injection. A reduced liver and spleen uptake was observed in vivo for batches with increased PEG amounts. Ex vivo quantification analysis reconfirmed this result. Additional ex vivo confocal microscopic experiments proved the previously found accumulation of polymeric nanocapsules in the ovaries [6; 166].
K Monitoring the in vivo efficiency of rhBMP-2 loaded microparticles

(X) Lochmann, A., Schädlich, A., Nitzsche, H., Metz, H., Schön, I., Schwarz, E., Mäder, K., Quantitative Monitoring of the in vivo Efficiency of rhBMP-2 loaded PLGA and PEG-PLGA Microparticles by means of Optical Imaging, CT and BT-MRI. (in preparation)

K 1. Summary

The Bone Morphogenetic Protein 2 (BMP-2) is important for the development of bone and cartilages. The recombinant human osteogenic protein (rhBMP-2) is used in the United States for the treatment of bone defects. After implantation, the rhBMP-2 protein induces bone formation. However, the protein is quite unstable which requires the administration of high doses. Therewith, the therapies are very expensive by what they are accessible only to a limited number of patients. Research is focused to reduce the rhBMP-2 doses while developing controlled parenteral protein release formulations.

Micro-particulate drug delivery systems which proved to be promising were developed [167]. In vivo fluorescence imaging was used to research the biodistribution as well as the pharmacological effect on bone formation. The protein was labeled with Rhodamine B to research the rhBMP-2 delivery [167]. In vivo fluorescence imaging was also used for tracking the rhBMP-2 induced calcification progress which is an important parameter of bone formation. Therefore, a NIR fluorescent calcium chelator was i.v. injected one day prior the measurements [167]. Comparable principles as used for the detection and visualization of the accumulation of nanoparticles in tumor tissues were used to visualize and quantify the calcification progress as well the distribution of the rhBMP-2. Fluorescence imaging was performed up to 12 weeks after rhBMP-2 application and confirmed the calcification effect for all investigated formulations [167].
Chapter VI: Fluorescence pre-clinical imaging – an overall discussion of results

L Potential capabilities and limitations of pre-clinical fluorescence imaging

The results of this work confirmed that in vivo fluorescence imaging is a promising technology for pre-clinical research of parenteral drug delivery systems. Based on the presented current results as well as on the data from literature it is expected that fluorescence imaging will have great impact to future drug development studies [168]. The studies revealed the broad range of potential applications of the method. Fluorescence imaging allowed monitoring and visualizing of body tissues as well as the assessment of molecular biodistribution processes. Results showed that the imaging modality has a high specificity in analyzing different and a high sensitivity in detecting even smallest fluorescence concentrations. This fact is in concordance with the general knowledge about optical imaging [169]. Plenty of open questions related to fundamental capabilities and limitations of fluorescence in vivo imaging were raised at the beginning of this research such as:

- What is the optical resolution?
- Does reflection and absorption influence the measurements and what is the maximum depth of imaging?
- Which amount of dye is needed?
- What is the reproducibility of the method?
- Is the reproducibility related to the signal to noise ratio?
- Which dyes can be used and which sensitivity do they have?

Experiments performed emphasized the capabilities and limitations of fluorescence in vivo imaging and complemented insufficient knowledge when using the technique as a pre-clinical imaging tool.
Major limitations of pre-clinical fluorescence imaging studies

It is known that a major limitation of fluorescence imaging is the lack of penetration depth of the light into body tissues [23; 88; 89]. The photon penetration into living tissue is highly dependent on its absorption and scattering properties [170]. Both, the scattering and the absorption of light are primarily related to the intensity and the wavelength of the excitation light and to the emission maxima of the fluorescence dye. This can result in low penetration depths of a few millimeters and thus highly influence the resolution of in vivo imaging records. The Maestro in vivo imaging system used within the current research is based on reflectance mode measurement principles and equipped with a broad beam xenon lamp. Laser systems can excite dyes to higher energy levels which enhances the underlying organ imaging. However, increased excitation laser power might also result in tissue photo damage. Another limiting factor is that laser systems do only excite certain wavelengths, which limits their use to a small number of special dyes.

As the Maestro imaging system uses a broad beam xenon lamp, the excitation intensity was default and the excitation light is filtered within the range between 445 nm and 760 nm by using six different optical filter sets. Thus, the optimal required wavelength range can be chosen individually for the dye used.

Another variable measurement parameter which mainly influences the measurements is the exposure time setting for each CCD frame. It was shown that lower acquisition exposure times reduced blurriness due to mouse breathing while they required high dye concentrations. Higher exposure times were needed if dye concentrations decreased over time. Simultaneously the noise level increased. Furthermore, it emerged that some experiments required constant exposure times over the whole series of experiments which required extensive evaluations and pretests [2].
**N Major challenges of pre-clinical fluorescence imaging studies**

**N 1. The choice of the correct dye**

When choosing appropriate dyes for an experiment, the advantages and disadvantages of different dyes were evaluated based on literature research and pretests. The selection was often hampered by the limited number of dyes for specific measurement issues. Using the Masetro *in vivo* imaging systems enabled due to a broad number of filters and of the multispectral measurement capabilities to measure various different dyes emitting at different wavelength ranges simultaneously.

As an example, this allowed measuring of two dyes emitting at different wavelength ranges within the PVA experiments. Therefore, the dyes TMR (emitting in the VIS range) and the NIR dye Alexa Fluor 750 were utilized. TMR allowed the differentiation of PVA accumulations in various fat tissues. The NIR dye facilitated the characterization of PVA elimination processes e.g. from the liver [1-3] as well as the measurement of the biodistribution in deep located body tissues, or in organs which are highly supplied with blood. NIR fluorophores have higher molar extinction coefficients. NIR light can deep penetrate organs due to considerably low absorption coefficients of tissues in the emitting region between 700 nm and 900 nm [79]. Therefore, the NIR dyes proved to be an optimal choice to study the biodistribution processes of drug delivery systems. NIR dye molecules covalently bound to PVA polymer chains permitted within performed *in vivo* studies imaging depths up to a few centimeters [1; 5; 6]. However, the overall use of NIR dyes was restricted. The numbers of NIR tracer molecules for special demands are either limited or even no NIR dyes are available for special requests e.g. to measure pH values or polarities. NIR dyes have commonly low quantum yields [171]. This required longer exposure times for pre-clinical measurements which increased the signal-to-noise ratio. At the same time, the number of pictures taken per image file had to be reduced, to kept measurement times in acceptable ranges and to avoid blurring of the images caused by mouse breath. Consequently, the lower number of single images reduced the spectral detail information of the emission fluorescence spectrum. Those appeared to be less characteristic [4]. This is enforced by the properties of the light and therewith the mechanism of light propagation in body tissues [130]. After excitation, the emitted
light is isotropically scattered in the tissue [131; 172]. The increased number of scattering events for NIR dyes in tissues reduced detectable information while spectra got smoother. This results in a loss of spectral information depending on the measurement depth [128]. Therefore, the exact localization of the emission light source was hampered.

Knowledge obtained within the PVA studies allowed preselecting dyes for all further in vivo studies. In the case of nanoparticle biodistribution studies it could be assumed that VIS dyes would have restricted the studies to analyze surface tissues. Thus NIR dyes had to be chosen to research the biodistribution, accumulation and elimination of nanoparticles in deep tissues and organs.

Also the way of incorporating the dye molecule into the drug delivery system has to be considered while choosing the best suitable one. Generally either physical entrapment or covalent labeling is the preferred technique. Whereas, dyes had to be labeled covalently to research the distribution in case of polymers they could be physically entrapped in nanocarriers.

Covalent labeling normally must be stable in in vivo surroundings. In addition it retrieves the risk that physicochemical properties e.g. of the polymer are influenced due to the general high molecular weights of the NIR dyes. Thus, the number of NIR molecules which were labeled to the polymer backbone was reduced to a minimum within PVA studies in order to preserve the properties of the PVA polymer [1].

For nanoparticle characterization NIR dye molecules were physically entrapped. Therefore, very hydrophobic dyes were required. Those cannot diffuse out of the drug delivery system until the carrier is degraded. The dialkylcarbocyanine dye DiR was chosen because of its high lipophilic properties [113-115] by what the dye is insoluble in aqueous media. Therefore a distinct leakage of the dye from the nanoparticles could be excluded [173; 174].

Nanoparticle studies proofed further that the emission spectra of NIR dyes are similar in vitro and in vivo. The penetration of emitted NIR fluorescence light through living tissue has no influence on the spectral emission profile [4]. Due to the above discussed loss of spectral information in NIR emission ranges, VIS fluorescent dyes were used if the detection of spectral changes was needed to characterize the drug delivery systems regarding physicochemical properties.

The detection of spectral changes was amongst others the basis for pH measurements in in situ forming implants as well as for pH and temperature measurements in tablets [4; 7; 8; 165]. Even smallest differences in the emitted fluorescence light spectra could be detected and used for extensive calculations.
However, based on the absorption and scattering properties of the light in the VIS wavelength range the measurements were limited to the near-surface areas. In case of performed in vivo pH measurements the detection limit was a few millimeters [7]. Summarized, it can be concluded that the choice of the correct dye depends on plenty individual experimental requirements and has to be considered previously.

N 2. Quantification – an impossible challenge in pre-clinical in vivo experiments?

In vitro, the fluorescence intensity is normally proportional to the concentration of the fluorophore and can be influenced e.g. by the spectroscopic properties of the emission light, the scattering effects of light and the technical equipment used. The magnitude of the in vivo emitted light strongly depends on a multiplicity of additional influencing factors. Many body components like haemoglobin and water but also chlorophyll in the fodder of the mice influences the emission of the light. Also parameters like the depth of the fluorescence source within the tissue, the density and homogeneity of the tissue itself as well as the absorption of the tissue influence measurements. Planar imaging systems like the Maestro in vivo imager which was used in the current work detect the fluorescence response which is projected to the surface of the living objects [175]. Quantification of the fluorophors as it is known from the nuclear medicine (determination of the percentage drug dose per respective organs) is at least not yet possible when fluorescence imaging is applied. In general, semi-quantitative data might be obtained using fluorescence imaging.

Within this research work, several approaches for the Maestro imaging system were investigated in order to emerge strategies allowing comparative semi-quantitative statements of different drug delivery systems. For instance, pharmacokinetic information like circulation time would allow better interpretation of the effectiveness of polymers and of different sized nanocarrier batches. Detailed pretests were necessary in order to investigate potential analyzes strategies. The techniques known from the intravital microscopy such as the fluorescence intensity measurements in blood capillaries in surface tissue regions [176; 177] were tried to adapt. However, even in the cases when it was possible to visualize blood vessels indicating the circulating polymers and nanoparticles [3; 4], the quantification approaches failed. Fluctuating fluorescence intensities from the blood vessels could not be detected because of the limited resolution of the captured fluorescence images. Also the evaluation of HPLC methods to determine the dye concentration in blood samples failed due the complexity of the disturbing blood
components and the limited blood volume available for analytic measurements. The combination of different visual analysis and calculation approaches using underlying data was a prerequisite for comprehensive characterization of drug delivery systems. Image analyses were performed for all experiments and allowed the \textit{in vivo} localization of analyzed dyes in the body [1; 3-7]. The visualization possibilities and the compared to other pre-clinical techniques like MRI or CT high resolutions of the images was another benefit of fluorescence imaging. Results like the detection of accumulated dye in the ovaries were only found while using the visualization functions [6]. In combination with \textit{ex vivo} results, studying the biodistribution, accumulation and elimination of processes was feasible. The Maestro imaging system enabled the monitoring of the multiple fluorescence probes for multispectral imaging approaches too [137; 178; 179]. Therewith, the spectral unmixing functionality enabled to visualize tumor tissues and accumulated nanoparticles simultaneously \textit{in vivo} [5]. This facilitated meaningful pre-clinical results while importantly reducing the number of animal experiments. The same multispectral measurement principles were also the basis for the visualization of changing pH distributions in \textit{in situ} forming implants [7]. The multispectral functionality allowed further the development of an imaging strategy that visualizes the local temperature allocation in tablets which occurred during the tablet pressing process [165].

Another imaging analysis technique used was the compare imaging tool. This analyzing possibility enabled the comparison of different images within a measurement series. Thus individual images taken over time can be correlated even if they have not the same scaling or intensities due to varied bit-depths, exposure times and binnings [180; 181]. Conclusions regarding the biodistribution over time were made based on the visualization results of the intensity differences in the component images [1; 3-5]. The same function enabled the visualization of the dye intensity change over time in an \textit{in situ} forming implant [6]. Therewith, statements regarding the micro-mobility in \textit{in situ} forming implants were possible. The findings indicated the accumulation of degradation products which was in accordance to published EPR results [161; 182].

Other data analyses were based on ROI principles. These were used to extract total and maximum intensities from isolated emission images. Therefore, the underlying data of the unmixed greyscale images were analyzed. The ROI in the size of the whole mouse or in the dimensions of tissues or organs of performed \textit{ex vivo} experiments were used. The total fluorescence intensity, calculated as the sum of all
fluorescent pixels within the ROI were assigned to a reference spectrum [181]. The maximum intensity reflects the intensity value of the pixel, having the highest intensity within the selected ROI. Both, the total and the maximum fluorescence intensities were exposure time weighted. This allowed comparing the data obtained at different exposure times, which was a prerequisite to measure at optimum, automatically defined measurement conditions. Thus the analyses made by means of total and maximum intensity calculations enabled to track the biodistribution of PVA for more than 180 days [3]. Comparative long-term studies between VIS and NIR labelled high molecular weight PVA provided detailed information about the biodistribution processes. The fluorescence emission of TMR was absorbed nearly completely by organs highly supplied with blood. Thus, the TMR signal was only detectable in the surface regions and analyses reflected only the accumulation in s.c. tissues. Due to the better optical penetration properties of NIR light, the accumulation in deeper tissues was investigated while analyzing total and maximum intensities of detected NIR signals. These semi-quantitative analyses underlined the importance to combine the optical properties of different dyes in order to obtain comprehensive pre-clinical information of the drug delivery system being researched. Further approaches were evaluated to characterize the in vivo fate of nanoparticles. As discussed above, resolution limitations of the CCD camera chip restricted the analysis of blood vessels in order to calculate blood half-life times. Therefore, the total signals of the body were measured at different time points. The calculated values reflect the sum of all s.c. dye signals (from blood vessels, upper parts of liver and spleen). They indicated that 50 % of the initially detectable nanoparticles were eliminated in about 45 minutes and about 75 % in 95 minutes after injection [4]. Tumor accumulations as well as nanoparticle enrichments in various tissues and organs were measured using PVA characterization principles. Detailed additional experiments were performed ex vivo to exclude interdependencies between the detection of fluorescence signals from overlying body tissues. Reproducible conditions like the arrangement of organs, the positioning in the Maestro imaging system, incident angle of the light, etc. were addressed while performing a different approach. Organs or respective organ parts in case of intestine and liver, were placed into a transparent 24-holes well-plate [4-6]. Performed ex vivo imaging results confirmed the nanoparticle accumulation in organs as already detected in vivo. The total and the maximum intensity analyses were used for ROIs defined in the sizes of the well-plate holes. The direct comparisons of total intensities proved to be challenging due to the diversity of dye loadings and dye allocation e.g. in different
nanoparticle batches [4]. A new calculation approach was developed as basis for further evaluations of size-dependent tumor accumulation studies with different nanoparticle batches [4]. This allowed recalculating the effects of varying dye loadings reproducible. To exclude the influence of different dye amounts, the ex vivo total intensity values were divided through the maximum intensity of the in vitro emission spectra of the nanoparticles. The studies proved that same tissues of different batches can be compared. Analyzing the maximum intensity values enabled the comparison of nanoparticle accumulation in organs of different sizes. Therewith, comparative in vivo and ex vivo analyses of the nanoparticle accumulation in xenograft tumor tissues could be performed [5].

Another novel calculation approach was used for the in vivo pH calculations of in situ forming implants. To get measurement files with more detailed information multispectral imaging cube sets were acquired in 2 nm steps with two different filter sets. Average emission spectra of several ROI were extracted from the measurement cubes. The new established calculation approach used three intensity values of the emission curve. This calculation eliminated the influence of both, varying dye concentrations as well as of different exposure times and allowed to calculate in vivo pH values reproducible. Furthermore, this approach reduced the influence of disturbing auto-fluorescence.

Altogether, the obtained results proved that the evaluated fluorescence imaging method is useful to semi-quantify fluorescence emissions in vivo. Recently developments were published which might enable better quantification of dyes in the future. Those improved systems are based on theoretical models of photon propagation as well as of absorption and scattering processes of the excitation and the emission light in tissues. The underlying mathematical calculations of those fluorescence imaging techniques would provide 3-D information in future. Examples are fluorescence-mediated tomography (FMT), optical projection tomography (OPT) and diffuse optical tomography (DOT) [183]. Those systems might enable the quantification of dyes independently from their depth in the body. First systems were already successfully applied in vivo to monitor tumor tissue, vascular volume fractions as well as for the quantification of pulmonary inflammation [184-187]. Meanwhile also prototype diagnostic NIR light applications for humans like mammography, retinal angiography, detecting of rheumatoid arthritis in hands and liver functional testing were developed [110; 188-190]. However, the mathematical models for the analysis and calculation of the optical tissue properties still require many efforts and additional extensive studies until those systems can be used.
Chapter VII: Summary and future perspectives

O Summary

Fluorescence imaging emerged in the early twenty-first century as a potential in vivo imaging technique to track fluorescence markers non-invasively. In the drug discovery research field of the pharmaceutical technology this method could allow to follow-up the distribution, accumulation and elimination processes of APIs or drug model substances in living organisms. In addition it is an excellent easy to use method to research physicochemical properties and in vivo behaviors of various drug delivery systems. Thus, fluorescence imaging conducted in early pre-clinical phases, could help to understand and optimize drug delivery systems. It is expected that next to the scientific benefit, fluorescence imaging can have also a major economic impact on cost saving due to the higher informative value of earlier and more precise pre-clinical experiments. However, new techniques possess new questions and challenges like: Which pharmaceutical technology research fields can benefit from fluorescence imaging? Which information can be obtained? Which limitations may hamper the application?

The Maestro imaging system as one of the first commercially available pre-clinical fluorescence imagers was launched to the market in the beginning of this research work. There was a lack of data and available knowledge about the application possibilities of this technique in order to characterize parenteral formulations. Therefore, the aim of this work was focused on evaluating in vivo and ex vivo imaging methods. In addition different analyzing approaches were developed to characterize parenteral formulations. Altogether, novel knowledge and an advanced understanding of the in vivo behavior of characterized complex drug carrier systems were provided.

The performed fluorescence imaging studies were focused on three different parenteral drug delivery systems: (a) water soluble polymer conjugates (publications I - III), (b) nanocarriers (publications IV - VI) and (c) in situ forming implants (publication VII). These were applied via three different administration routes: (a) i.p., (b) i.v., and (c) s.c. [1-7]. In addition the gained information from performed pre-
clinical in vivo studies was used to characterize also (d) alternative drug delivery systems (publication VIII) [8].

All in vivo experiments were complied in accordance to the animal protection standards as stated in the guideline from the animal care and use committee of Saxony-Anhalt and were officially approved.

(a) Pre-clinical in vivo studies of PVA were successfully performed to investigate the long-term in vivo fate after i.p. injection. PVA was either covalently labeled with the VIS dye TMR (fluorescent in the range of visible light) or with the NIR dye Alexa Fluor 750. Using fluorescence imaging and two in their characteristics completely different dyes allowed performing comprehensive studies of the in vivo biodistribution of PVA. New findings are mainly related to the in vivo distribution, accumulation and the release from accumulated organs and tissues.

The characteristic emission spectra of TMR and using the unmixing functions of the Maestro imaging systems enabled to visualize the blood vessels due to long circulating PVA molecules. Also the accumulation of PVA in fat tissues has been identified with the use of the imaging system for the first time. Additionally the non-invasive differentiation between the accumulation of PVA in abdominal fat and in s.c. fat tissue has been shown. The amounts of accumulated PVA were analyzed and the further release rates were tracked for more than 6 months. Using different analyzing approaches a distinction between the release in female and in male mice was possible. Further ex vivo studies confirmed in vivo findings of PVA accumulation in fat tissues. Additional applied confocal microscopy permitted to follow the PVA accumulation up to cellular levels. Thereby, PVA enrichment between fat cells has been verified.

In vivo experiments performed non-invasively with NIR labeled PVA enabled studying the biodistribution of PVA also in deeper tissues and in organs which are highly supplied with blood. The combination of the advantages of both dyes facilitated studying the PVA elimination pathways. During the first days the PVA has been found to be mainly excreted via the urine. In addition, the long-term elimination of PVA was followed in all tissues where PVA was accumulated. Longest, NIR labeled PVA was detectable in the liver. After up to 9 months the concentration dropped below the detection limit. This suggested a complete elimination of water soluble high molecular weight PVA from the body.

A major challenge in performing pre-clinical in vivo experiments is to get a maximum of information while reducing the stress for each animal during an experiment as well
as minimizing the total number of animal experiments. The method of fluorescence imaging fulfills these both conditions. Within this research it has been shown that PVA could be non-invasively followed from the point of injection until it has been eliminated from the organism while miscellaneous information was obtained simultaneously. This enabled to reduce the number of terminal animal experiments to a minimum.

(b) Additional pre-clinical in vivo studies were conducted to access the in vivo fate of nanoparticles, their potential to be used in targeted cancer therapy and possible toxicity risks which may occur during their administration. Thorough particle size measurement studies were performed in order to justify size-dependent biodistribution conclusions afterwards. Experiments with AF4/MALLS, PCS and TEM showed that particle sizes of all used batches were narrow distributed within predefined size ranges (between 100 nm and 200 nm).

The biodistribution of NIR nanoparticles was followed for several days in vivo and verified ex vivo. In the bloodstream circulating NIR loaded nanoparticles were detectable in vivo for up to 6 hours. A new ex vivo imaging procedure was evaluated to get comparative biodistribution data of nanoparticles in intestine, fat, uterus with ovary, liver, gall bladder, lung, spleen, kidneys and heart. These extensive ex vivo studies were performed to enable size dependent justifications of the in vivo fate. Based on the data acquired within the research a new calculation method has been developed. This allowed time-dependent comparisons of total and maximum fluorescence intensities of different batches with varying intensities. This approach was a pre-requisite to research the influence of varying particle sizes on the biodistribution and the potential tumor accumulation ability in future.

Nanoparticle biodistribution studies were performed to investigate the potential use in cancer therapy. The use of non-invasive multispectral fluorescence imaging allowed for the first time to combine tumor visualization of DsRed2 expressing HT29 xenografts with simultaneous tracking of the nanoparticle accumulation. In addition size dependent accumulation studies in human colon carcinoma (HT29) and ovarian carcinoma (A2780) xenografts have been performed. The studies showed that all tested nanoparticles were accumulated in both tumor models whereas different sized nanoparticle batches were most effective for HT29 as well as for A2780 xenografts. Ex vivo studies showed different accumulation pattern. Slightly bigger nanoparticles were most effective and enriched primarily in specific necrotic regions in the tumor...
center of HT29 tumors. This was confirmed by confocal microscopy. Nanoparticles were visualized between fluorescent, DsRed2 expressing HT29 cells. Light microscopic studies of thin section slides of tumor tissue confirmed the accumulation in necrotic tissues for HT29 xenografts.

No centralized nanoparticle accumulation was observed for A2780 tumors. Slightly smaller sized nanoparticles were most effective. This led to the conclusion that vascular permeation for A2780 and accumulations in necrotic areas for HT29 were the preferable mechanisms of nanoparticle accumulation.

In vivo experiments identified also high local, punctual enrichments in parts of the ovaries for all tested nanoparticle batches. Within ex vivo experiments it has been found that the total fluorescence signals from overall ovarian tissues were rather low compared to other tissues with verifiable nanoparticle accumulation. Ex vivo fluorescence imaging and confocal microscopy studies enabled to localize the accumulation. This effect has been confirmed in further experiments in a second mouse species (BALB/c) and in Wistar rats. Same accumulation pattern were found for two other tested nanocarrier systems, for nanocapsules and nano-sized lipid emulsions. It has been concluded that an accumulation of nanocarriers was most likely in the fast-growing corpus luteum and is related to the enhanced permeability and retention effect known for tumor tissues.

The presented studies demonstrated that fluorescence imaging allowed investigating the in vivo fate of drug delivery formulations while imaging fluorescent tissues at the same time. The experiments can be the basis for future pre-clinical studies to determine pharmacokinetic parameters next to the therapeutic success. The gathered measurement principals allow measuring the nanoparticle biodistribution next to the tumor localization and the detection of changing tumor sizes.

(c) Pre-clinical in vivo studies of in situ forming implants were performed to characterize both changing pH values and dye release profiles of PLGA implants. Thorough preliminary in vitro and ex vivo fluorescence imaging experiments have been performed. The effect of external influencing factors like system, components, measurement depths etc. as well of varying measurement conditions (varying exposure times, resolutions, filter settings) were assessed.

A novel non-invasive in vivo pH measurement method for fluorescence imaging was established. This allowed for the first time to perform non-invasive measurements and visualizations of the micro pH shift in in situ forming PLGA implants. The pH decrease was measured non-invasively for more than 3 weeks and confirmed
Chapter VII: Summary and future perspectives

*ex vivo.* The results are in good accordance to other *in vitro* EPR and *in vitro* confocal laser scanning microscopy studies. The comparison of the studies has been shown that the pH change occurred *in vivo* slower than *in vitro.* Measuring the fluorescence intensity of the dye allowed further analyzing the solvent/non-solvent exchange process during implant solidification and to draw conclusions regarding the mobility of dye molecules in the implant. The physically entrapping of the hydrophilic dye facilitated also to research the release profiles from the implant.

The experiments showed that fluorescence imaging serves as valuable tool for pre-clinical studies. The technique enabled the non-invasive detection of pH values and the characterization of parenteral drug formulations.

*(d)* The knowledge obtained from the current pre-clinical *in vivo* research studies has been applied to characterize also other non-parenteral drug delivery systems. The conducted *in vivo* pH measuring principles were successfully used to monitor non-destructively pH gradients in swelling multi-layer tablets. As a prerequisite for *in vitro* pH measurements in tablets additional pretests were performed to access the influence of e.g. excipients, different dyes and dye concentrations. Imaging experiments allowed to measure and to visualize the spatial pH distribution in the tablet surface across the swelling process. It has been shown that the incorporation of internal buffer substances and the choice of excipients strongly influenced the pH in the swelling layer. The pH measurement results obtained in the current research were comparable with other performed pH measurement techniques: the EPR spectroscopy and the usage of pH indicator substances. Results showed the respective different potentials of each of the methods.

A comparable approach was used to monitor the temperature distribution in tablets, caused during the tableting process. A dye mixture was identified which changes irreversible the intensity of two emission maxima when exposed to temperatures between 40 °C and 80 °C. Extensive temperature calibration tests were successfully performed. Due to limitations of the Maestro software, a manual Microsoft Excel based procedure for temperature analysis was evaluated. The temperature which occurred during the tableting was five-fold determined for six mixtures and for three tableting processes. The analysis showed that the maximum observed temperature varied depending on used excipients, the humidity of the excipients and to some extent on the applied tableting conditions.
Obtained fluorescence *in vivo* imaging knowledge could also be used to characterize other parenteral drug delivery systems non-invasively *in vivo*. As part of the work also nanocapsules were thorough characterized *in vivo* and *ex vivo*. The established methods were further adopted to study the biodistribution and the *in vivo* efficiency of rhBMP-2 loaded microparticles.

The results of this research work demonstrated the potential applications of the pre-clinical fluorescence *in vivo* imaging method in the pharmaceutical technology research field. It has been shown that this technique offers a comprehensive imaging platform for the characterization and analysis of multiple *in vivo*, *ex vivo* and *in vitro* issues. Applied to small animals *in vivo*, this technique provided superior monitoring and visualization possibilities of drug delivery systems.

The presented new multispectral fluorescence imaging approaches serve as the basis for the characterization of other parenteral drug delivery systems. Thus this work can contribute to the better understanding of *in vivo* biodistribution processes in future.
Future perspectives

Based on the research findings presented within this cumulative thesis, new scientific questions emerged which should be addressed in future pre-clinical multispectral fluorescence imaging experiments. Briefly, further research should be focused on:

1. **Knowledge transfer to characterize other parenteral formulations**
   - Evaluated fluorescence imaging methods should be applied and broadened to characterize other parenteral formulations regarding their *in vivo* fate.

2. **Correlation of nanocarrier *in vivo* characterization and use for cancer treatments**
   - Further multispectral studies with fluorescent tumor tissues and fluorescent nanocarriers should be carried out simultaneously to investigate the accumulation behavior in more detail.
   - For the visualization of metastases in the body of small animals it should be tried to establish xenograft tumor models with fluorescent emissions in the dark red or even NIR wavelength area.
   - Size dependent tumor accumulation studies should be extended to other xenograft tumor models in order to get detailed understanding of the relationship between nanocarrier sizes and the tumor accumulation behavior.

3. **Research the accumulation of nanocarriers in ovaries**
   - Detailed investigations on the influence of size, surface structure and surface charge should be performed using various other nanocarrier systems.
   - To investigate dye and potential API polarity specific accumulations, other dyes should be incorporated and covalently bound to the nanocarriers.
   - The accumulation capabilities of high molar mass polymers which are covalently NIR labeled should be investigated.

4. **Implementation of *in vivo* pH measurements**
   - Visualization and measurement of pH values in tumor tissues should be conducted based on the knowledge obtained.
Q German summary


Hierfür wurden drei parenterale Arzneistoffträgersysteme: (a) wasserlösliche Polymere (Publikationen I - III), (b) Nanopartikel (Publikationen IV - VI) und (c) sich in situ bildende Implantate (Publikationen VII) ausgewählt welche auf drei
verschiedenen Applikationsarten injiziert wurden: (a) i.p., (b) i.v. und (c) s.c.) [1-7]. Es ist bekannt, dass das in vivo Verhalten aller drei Systeme sehr komplex ist und damit nur schwer in in vitro Experimenten erforscht werden kann. Des Weiteren wurde das in den in vivo Studien erlangte Wissen genutzt, um andere Arzneistoffträgersysteme zu charakterisieren (d) [8].
Alle Tierversuche innerhalb dieses Forschungsprojektes sind von der Tierschutzkommission des Landes Sachsen - Anhalt genehmigt und unter Beachtung deutscher Tierschutzgesetze durchgeführt worden.

Das Emissionsspektrum des NIR - Farbstoffes ist im Vergleich zum TMR - Spektrum weniger charakteristisch, so dass aus den aufgenommenen Bilddateien weit weniger spektrale Informationen abgeleitet werden konnten. Während der Durchführung von
in vivo Experimenten mit dem NIR - gelabelten PVA konnte aber Dank der Emission im NIR Bereich die Verteilung der PVA Moleküle in tiefer liegenden Geweben sowie in stark durchbluteten Organen nicht-invasiv verfolgt werden. Die Vorteile beider Farbstoffe konnten ebenfalls zur Untersuchung von Ausscheidungsprozessen genutzt werden. In den ersten Tagen wird PVA demnach hauptsächlich mit dem Urin ausgeschieden was eine bereits publizierte ESR Studie an Kaninchen bestätigt [34]. Weiterhin wurde mit der NIR markierten PVA - Charge die Eliminierung des Polymers aus jenen Geweben verfolgt, in denen sich das Polymer anreicherte. Am Längsten war der NIR Farbstoff in der Leber detektierbar. Aber auch hier fiel die Intensität des Farbstoffes nach bis zu 9 Monaten unter die Detektionsgrenze. Daraus ließ sich ableiten, dass das hochmolekulare PVA über die Zeit wohl vollständig aus dem Körper ausgeschieden wird.

Mittels der Fluoreszenzbildgebung und der Untersuchung zweier in ihren spektralen Eigenschaften völlig unterschiedlicher Farbstoffe war es möglich, umfangreiche Untersuchungen hinsichtlich des in vivo Verteilungsverhaltens von PVA durchzuführen. Daraus ergaben sich neue Erkenntnisse bezüglich der in vivo Verteilung des Polymers, sowie dessen Anreicherung und Freisetzung aus Organen und Geweben.


(b) Weiterhin wurden präklinische Studien durchgeführt, die die Verteilung von Nanopartikeln im Körper untersuchen, um schließlich Rückschlüsse bezüglich der Anwendbarkeit der hergestellten Partikel in der Tumortherapie ziehen zu können. Außerdem sollte herausgefunden werden ob eine potentielle Toxizität von ihnen ausgehen könnte. Um nun auf Basis der durchgeführten Fluoreszenzmessungen größenabhängige Aussagen treffen zu können, sind vor Studienbeginn umfassende Partikelgrößenmessungen durchgeführt worden. Die AF4/MALLS, PCS und TEM Messungen bestätigten, dass die Partikelgrößen aller gemessenen Chargen eng

Zusammenfassend kann festgestellt werden, dass es mittels der Fluoreszenzbildgebung möglich ist *in vivo* Verteilungsprozesse von Arzneistoffformulierungen darzustellen. Damit ist es in zukünftigen präklinischen Studien möglich die Pharmakokinetik und den Therapieerfolg, also beispielsweise die Reduktion der Tumorgröße, gleichzeitig zu bestimmen.

der optischen Charakterisierung mittels pH Indikatoren vergleichbar. Dabei wies jede der genutzten Methoden unterschiedliche Vor- und Nachteile auf und leistete zur Beantwortung verschiedener Fragestellungen einen wichtigen Beitrag.


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Supplemental material

This cumulative dissertation contains the following research articles.

(a) Investigation of the in vivo fate of a water soluble polymer

(I) Title: Noninvasive in vivo monitoring of the biofate of 195 kDa poly(vinyl alcohol) by multispectral fluorescence imaging.
Authors: Schädlich, A., Naolou, T., Amado, E., Schöps, R., Kressler, J., Mäder, K.
© Copyright (2011) American Chemical Society.
http://dx.doi.org/10.1021/bm200899h

(II) Title: In-vivo studies on intraperitoneally administrated poly(vinyl alcohol).
Authors: Jiang, Y., Schädlich, A., Amado, E., Weis, C., Odermatt, E., Mäder, K., Kressler, J.
© Copyright 2010 Wiley Periodicals, Inc.
http://dx.doi.org/10.1002/jbm.b.31585

(III) Title: Tracking the in vivo fate of high molar mass poly(vinyl alcohol) using multispectral fluorescence in vivo imaging.
Authors: Schädlich, A., Jiang, Y., Kressler, J., Mäder, K.
Reprinted with permission from Scientifically Speaking News (Controlled Release Society) 27 (2), Andreas Schädlich, Yanjiao Jiang, Jörg Kressler, Karsten Mäder, Tracking the in vivo fate of high molar mass poly(vinyl alcohol) using multispectral fluorescence in vivo imaging. (2010), 15-16.
(b) Characterization of nanocarriers and their potential usage in cancer therapy

(IV) Title: How stealthy are PEG-PLA nanoparticles? An NIR in vivo study combined with detailed size measurements.

Authors: Schädlich, A., Rose, C., Kuntsche, J., Caysa, H., Mueller, T., Göpferich, A., Mäder, K.


http://dx.doi.org/10.1007/s11095-011-0426-5

(V) Title: Tumor accumulation of NIR fluorescent PEG PLA nanoparticles: impact of particle size and human xenograft tumor model.

Authors: Schädlich, A., Caysa, H., Mueller, T., Tenambergen, F., Rose, C., Göpferich, A., Kuntsche, J., Mäder, K.


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(VI) Title: Accumulation of nanocarriers in the ovary: A neglected toxicity risk?

Authors: Schädlich, A., Hoffmann, S., Mueller, T., Caysa, H., Rose, C., Göpferich, A., Li, J., Kuntsche, J., Mäder, K.


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http://dx.doi.org/10.1016/j.jconrel.2012.02.012
(c) Characterization of *in situ* forming implants for potential controlled API release

(VII) **Title:** Long-term *in vivo* pH measurements of *in situ* forming PLGA implants using multispectral fluorescence imaging.  
**Authors:** Schädlich, A., Kempe, S., Mäder, K.  

(d) Investigating alternative application fields of fluorescence imaging

(VIII) **Title:** Monitoring of internal pH gradients within multi-layer tablets by optical methods and EPR imaging.  
**Authors:** Eisenächer, F., Schädlich, A., Mäder, K.  
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[http://dx.doi.org/10.1016/j.ijpharm.2010.10.010](http://dx.doi.org/10.1016/j.ijpharm.2010.10.010)
Noninvasive in Vivo Monitoring of the Biofate of 195 kDa Poly(vinyl alcohol) by Multispectral Fluorescence Imaging

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Abstract: A comprehensive knowledge of the in vivo fate of polymers is essential for their potential application in humans. In this study, the body distribution, accumulation, and elimination processes of intraperitoneally (ip) administered poly(vinyl alcohol) (PVA) in mice were investigated in detail. Two derivatives of PVA (195 kDa) having covalently bound fluorescent dye labels were synthesized and used to follow PVA in vivo by noninvasive multispectral fluorescence imaging over several months. Detailed ex vivo fluorescence imaging was performed additionally and combined with tissue accumulation studies using confocal microscopy. Filtration and confocal imaging at appropriate synthetic membranes, used as models for glomerular filtration, confirmed a considerable PVA permeation. This investigation yields new scientific findings about the fate of PVA in vivo. PVA accumulated in fat tissue at high levels, which suggests that PVA is suitable not only for abdominal surgeries but also for controlled release applications after ip or subcutaneous injection.

Introduction

Biocompatible polymers are being extensively studied for numerous pharmaceutical applications in the form of microspheres, nanoparticles, in situ forming implants, coatings and for protein delivery or as a plasma expander. Poly(vinyl alcohol) (PVA) is already used in various pharmaceutical and biomedical applications as well as in biotechnology and food chemistry.1,2 It is a water-soluble, nonionic, nontoxic, nonimmunogenic synthetic polymer. It is relatively inert in body fluids, and it is approved in several market products by the Food and Drug Administration (FDA).2–5 The properties of PVA are mainly influenced by its molar mass and the degree of hydrolysis of the precursor polymer poly(vinyl acetate). PVA has a high content of hydroxyl groups, good mechanical strength, high elasticity, and weak protein adsorption.5–9 It is reported that PVA hydrogels have high water contents and excellent mechanical properties, because of which they are ideally suited for soft tissue engineering and for the immobilization of molecules and cells.5–9 PVA is also used as articular cartilage, for catheters, and as artificial skin.5,10,11 PVA-based hydrogels are being extensively studied for drug delivery applications, too.12–14 Because of its excellent film forming, emulsifying, and adhesive properties, PVA hydrogels are considered for accelerating wound healing and preventing postoperative adhesions.15–17 which are internal scars affecting patients after abdominal surgeries.17,18 Polymeric membranes currently used to prevent adhesions are among others oxidized, regenerated cellulose membranes (Interceed, Ethicon 360), hyaluronic acid—carboxymethylcellulose membranes (Seprafilm, Genzyme), and expanded polytetrafluoroethylene membranes (Preclude, Gore).19–22 Such polymeric membranes have several drawbacks. Some of them are not biodegradable or cannot be reabsorbed after peritoneal healing. A major disadvantage of membranes is that they cannot be applied in every surgical site. In contrast, gels or liquid solutions can easily be injected over the wound and also be applied to less accessible areas. PVA has already been proven as a promising alternative. Also, the additional binding of drug molecules to the PVA backbone is easily achieved and would allow simultaneous drug therapy at the site of application. High-molar mass PVA must be used to prolong the remaining time at the place of application.16 Some studies showed that PVA polymers with molar masses of 125 or 195 kDa are the most suitable.15,16,21 In spite of these potential uses, the detailed in vivo fate of high-molar mass PVA after intraperitoneal (ip) application is still unexplored. Until now, only a few studies deal with PVA elimination pathways.16,22–24 Because PVA biodegradation is limited to bacteria and fungi and has not been found in humans, the polymer is eliminated without being changed. Recent studies demonstrated that ip administered PVA up to 195 kDa can be excreted through the kidneys without renal glomeruli being damaged.16,22 This was...
observed even though the molar mass and the hydrodynamic radius \( R_h \) of expected PVA spheres (\( \sim 13 \text{ nm} \)) are far larger than the reported limits (\( R_h < 6.5 \text{ nm} \) or a molar mass of \( <30-69 \text{ kDa} \)) for glomerular protein and polymer filtration.\(^{24,27}\) This fact can be explained by the flexibility of the PVA chains.

In this study, extensive long-term in vivo investigations were performed to follow the fate of PVA in a noninvasive way. PVA (195 kDa) was labeled either with a rhodamine dye or with Alexa Fluor 750, a near-infrared (NIR) dye. Both dyes possess different imaging characteristics, which allowed complementary extensive in vivo studies over several months. The obtained distribution, accumulation, and elimination behaviors were complemented with ex vivo experiments, including confocal microscopy studies to follow the fate of PVA at the cellular level. To gain a better understanding of the PVA in vivo elimination processes, in vitro filtration experiments with commercial available polyethersulfone (PES) membrane filters were performed. The membranes filters were washed with nominal molecular mass cutoffs (NMMC) of 30 and 50 kDa and nominal pore sizes, which were much smaller than the hydrodynamic diameter of PVA (195 kDa) molecules.

## EXPERIMENTAL SECTION

### Materials

PVA (Mowiol 56-98, Kuraray) had an average molar mass \( M_n \) of 195 kDa (4300 repeating units; \( n \)) polydispersity index (PDI) of 2.3, and a degree of hydrolysis of 98.4 mol % (data provided by Kuraray). Dry dimethyl sulfoxide (DMSO, 99.5%), dimethyl sulfoxide (DMSO, 99.5%), dimethyl sulfoxide (DMSO, 99.5%), dimethyl ether, dichloromethane (99.9%), methanol (99.5%), and p-toluenesulfonyl chloride were purchased from Sigma-Aldrich. For PVA synthesis, tetramethylrhodamine 5-carboxylic acid (TMR) and Alexa Fluor 750 were obtained from Invitrogen. Dry dimethyl sulfoxide (DMSO, 99.5%) and sodium bicarbonate were purchased from Sigma-Aldrich. For PVA synthesis, tetramethylrhodamine 5-carboxylic acid (TMR) and Alexa Fluor 750 were obtained from Invitrogen.

### Synthesis of Fluorescent Dye-Labeled PVA

For the in vivo fluorescence studies, the PVA backbone was labeled either with TMR (\( \lambda_{	ext{em}} = 577 \text{ nm} \)) (named PVA-TMR) or with the NIR dye Alexa Fluor 750 (\( \lambda_{	ext{em}} = 780 \text{ nm} \)) (named PVA-NIR).

**PVA-TMR Labeling.** PVA was labeled as described previously.\(^{22}\) Briefly, the reactive fluorescence TMR dye and the PVA were dissolved in anhydrous DMSO (2.2/1 PVA/TMR molar ratio). The reaction was conducted at 80 °C for 12 h. The final labeled PVA (PVA-TMR) with a stable urethane bond\(^{28}\) was freeze-dried and stored in a desiccator, protected from light. The final degree of functionalization with TMR was 0.01 mol %. Thus, it can be assumed that every second polymer chain was labeled. The fluorescence emission maximum was determined to be 577.3 nm and is not shifted compared to that of pure TMR.

**PVA-NIR Labeling.** For PVA-NIR synthesis, five different steps were necessary (see Figure 1).

1. **Decrystallization of PVA.** This procedure was necessary to convert crystalline fractions to an amorphous state that is indispensable for conducting the tosylation reaction. Functional groups in the crystalline polymer would be inaccessible, and the chemical reaction would be limited to the amorphous region.\(^{29}\) PVA crystals were decrystallized using a water/methanol system. PVA (4 g) was dissolved at 70 °C in 50 mL of deionized water. The solution was allowed to cool to room temperature, and the polymer was precipitated into 300 mL of methanol. The resulting polymer was dried under vacuum at 70 °C for 1 day.

2. **Tosylation of PVA.** The reaction was performed according to the method of Ikeda et al.\(^{29}\) PVA (1 g) was swollen in 25 mL of pyridine (100 °C, 2 h). One gram (0.25 equiv with respect to the hydroxyl group) of p-toluenesulfonyl chloride was added at 0 °C. The reaction was conducted at room temperature (24 h). Afterward, the polymer was precipitated into methanol (400 mL), washed with methanol, and dried at room temperature under vacuum (24 h). \(^1\)H NMR calculation analysis indicated a tosylation ratio of 0.68% of the hydroxyl groups.

3. **Azidation of Tosylated PVA.** The azidation, already reported by Gacal et al.,\(^{30}\) of tosylated PVA (0.9 g) by addition of sodium azide (NaN\(_3\), 0.52 g) was performed in 50 mL of DMF at 60 °C for 60 h. The product (PVA-N\(_3\)) was precipitated in 350 mL of dimethyl ether and washed with 700 mL of deionized water. The resulting \(^1\)H NMR spectrum showed complete disappearance of all tosy1 signals. The azide group was indicated by infrared spectroscopy.

4. **Amination of PVA-N\(_3\).** Amination was achieved via the Staudinger reaction. PVA-N\(_3\) (150 mg) was dissolved at 90 °C in DMSO (25 mL). After the sample cooled down to 24 °C, triphenylphosphine (1-Ph\(_3\)P) was added (14 mg), and the solution was stirred for 48 h. Deionized water (5 mL) was added, and the reaction was stopped after 24 h. PVA-N\(_3\) was precipitated in methanol. The resulting IR spectrum showed the complete disappearance of azide groups.

5. **Conjugation with NIR Dye.** This was performed by reacting PVA-N\(_3\) with the amine-reactive Alexa Fluor 750 carboxylic acid, succinimidyl...
In vivo measurements were taken using the Maestro in vivo fluorescence imaging system (Cambridge Research & Instrumentation) and the Maestro software (version 2.10). A C-mera-type 100 W xenon lamp with 5600 K, a green filter set for PVA-TMR (excitation range, 503–555 nm; emission, 580 nm long-pass), and a deep red excitation filter set for PVA-NIR (excitation range, 671–705 nm; emission, 750 nm long-pass) were used. The imaging proceeded in 2 nm steps for PVA-TMR and 10 nm steps for PVA-NIR. Exposure times were automatically set to optimal values. For time-dependent measurements, primarily determined exposure times were used for all further images. The in vivo reference spectra for PVA-TMR and PVA-NIR were generated via extraction of a sc in vivo dye signal from the chest area, 54 h after the injection of labeled PVA. This sc PVA-TMR or PVA-NIR signal consisted of dye emission and autofluorescence, due to molecules in tissues and fodder that can act as biochemical sources of fluorescence.34–36 For exact fluorescence imaging measurements, the autofluorescence was isolated from the mixed sc spectra by the manual computing software function. The detected autofluorescence signal was grabbed from an untreated mouse and measured under the same conditions. Using the reference spectra (isolated PVA-TMR, PVA-NIR and the autofluorescence as a background signal) allowed us to unmix and further segment the measured cubes.35–37 In the case of PVA-TMR, a further differentiation between subcutaneous [in the following named PVA-TMR (sc)] and deeper emitted signals [named PVA-TMR (ip)] was possible. To determine the PVA-TMR (ip) spectra, a signal from the ventral fat area (as overlay from background, ip, and sc signals) was manually computed using both pure sc (grabbed at the chest area) and background autofluorescence signals. Computed PVA-TMR (sc), PVA-TMR (ip), or PVA-NIR and corresponding background spectra allowed generation of monochrome images for each respective emission spectrum. These grayscale images were further calculated to obtain intensity-weighted incremental jet color images. Thereby, pixels with maximal intensities were set to dark red and pixels with no fluorescence to black. Between those two colors, there was a gradation from red to orange, yellow, light blue, and dark blue. All in vivo PVA-NIR images of a series of measurements were analyzed together (‘compare imaging’ function), correlating them to respective exposure times and using the incremental jet color profile simultaneously for all images. For quantitative analysis, an area of interest (ROI) in the size of the whole image was used to extract total and maximal intensities from isolated PVA-TMR (sc), PVA-TMR (ip), or PVA-NIR channels.

Ex Vivo Imaging and Analysis. Excised tissues (abdominal fat, kidney fat, skin, kidneys, liver, and spleen) of mice, sacrificed after defined periods of time (3 days and 3 months), were imaged with the Maestro in vivo imaging system. To ensure reproducible conditions like position and incident angle of the light, the organs were placed into a 24-hole well plate. The imaging setup was in accordance with in vivo measurements. Using emitted PVA-NIR signals allowed the calculation of corresponding jet color images, based on above-described compare imaging function.

Ex Vivo Confocal Imaging. Leica TCS SP2, as described for in vivo confocal imaging, was also used for confocal tissue imaging. The microscope was equipped with a 40× Plan Apo oil immersion objective. PVA-TMR was excited with a 543 nm laser. Emitted fluorescence was detected in the bandwidth from 555 nm to the upper limit. To image ex vivo samples, fat tissue was excised and cut into pieces that were as thin as possible into small panels (approximately 0.5 mm), using a racer plate. These were slightly pressed between two cover slides. Abdominal fat from an untreated mouse was incorporated for 3 min with 2 μL PVA-TMR, washed (20 min in purified water), and imaged.

RESULTS AND DISCUSSION

Synthesis of Dye-Labeled PVA. PVA was successfully labeled with two fluorescent dyes (TMR or Alexa Fluor 750) having ester dye. PVA-NH₂ (40 mg) and a 0.13 M sodium bicarbonate buffer solution (2.5 mL) were dissolved at 70 °C. The NIR Alexia Fluor 750 dye (1 mg) was dissolved in DMSO (100 μL) and slowly added to the PVA-NH₂ solution. After being stirred for 24 h at room temperature (24 °C), in the dark, the solution was dialedyzed (3500 g/mol cutoff dialysis membrane) against DMSO. The DMSO was replaced daily until no free dye was detected by fluorescence spectroscopy. Dialysis was continued to exchange DMSO against deionized water (2 days and 4 times). The final solution was freeze-dried, and a yield of 28 mg of PVA-NIR was obtained.

Pressure Filtration. Aqueous PVA solutions (100 mL, 0.1–1.0 mg/mL) were filtered at 21 or 37 °C and 1 bar of transmembrane pressure with a sterilized Amicon ultrafiltration cell (Millipore Corp.) with a K50 Omega filter disk (NMMC of 50 kDa, ∼5 nm pore size, 28.7 cm² filtration area) from Pall Corp.; 60 mL of each filtrate were collected and dried by evaporation to a constant weight. The concentration of the filtrate was calculated from the PVA mass and filtration rates from each filtration time.

Gel Permeation Chromatography (GPC). For molar mass distribution control, dried and redissolved filtrate (60 μL, collected over approximately 50 min) and retentate (40 mL) samples from pressure filtration (0.5 mg/mL PVA) were checked against a fresh prepared PVA solution with GPC max (Viscotek GmbH) by refractive index (RI) detection. Hydroxyl starch (HES) standards were used for calibration.

In Vivo Imaging and Analysis. All in vivo experiments complied with the standards for the usage of animal subjects as stated in the guideline from the animal care and use committee of Saxony Anhalt. In vivo measurements were taken using the Maestro in vivo fluorescence imaging system (Cambridge Research & Instrumentation) and the Maestro software (version 2.10). A C-mera-type 100 W xenon lamp with 5600 K, a green filter set for PVA-TMR (excitation range, 503–555 nm; emission, 580 nm long-pass), and a deep red excitation filter set for PVA-NIR (excitation range, 671–705 nm; emission, 750 nm long-pass) were used. The imaging proceeded in 2 nm steps for PVA-TMR and 10 nm steps for PVA-NIR. Exposure times were automatically set to optimal values. For time-dependent measurements, primarily determined exposure times were used for all further images. The in vivo reference spectra for PVA-TMR and PVA-NIR were generated via extraction of a sc in vivo dye signal from the chest area, 54 h after the injection of labeled PVA. This sc PVA-TMR or PVA-NIR signal consisted of dye emission and autofluorescence, due to molecules in tissues and fodder that can act as biochemical sources of fluorescence.34–36 For exact fluorescence imaging measurements, the autofluorescence was isolated from the mixed sc spectra by the manual computing software function. The detected autofluorescence signal was grabbed from an untreated mouse and measured under the same conditions. Using the reference spectra (isolated PVA-TMR, PVA-NIR and the autofluorescence as a background signal) allowed us to unmix and further segment the measured cubes.35–37 In the case of PVA-TMR, a further differentiation between subcutaneous [in the following named PVA-TMR (sc)] and deeper emitted signals [named PVA-TMR (ip)] was possible. To determine the PVA-TMR (ip) spectra, a signal from the ventral fat area (as overlay from background, ip, and sc signals) was manually computed using both pure sc (grabbed at the chest area) and background autofluorescence signals. Computed PVA-TMR (sc), PVA-TMR (ip), or PVA-NIR and corresponding background spectra allowed generation of monochrome images for each respective emission spectrum. These grayscale images were further calculated to obtain intensity-weighted incremental jet color images. Thereby, pixels with maximal intensities were set to dark red and pixels with no fluorescence to black. Between those two colors, there was a gradation from red to orange, yellow, light blue, and dark blue. All in vivo PVA-NIR images of a series of measurements were analyzed together (‘compare imaging’ function), correlating them to respective exposure times and using the incremental jet color profile simultaneously for all images. For quantitative analysis, an area of interest (ROI) in the size of the whole image was used to extract total and maximal intensities from isolated PVA-TMR (sc), PVA-TMR (ip), or PVA-NIR channels.
different, well-separated fluorescence emission maxima. The dyes were linked to the PVA backbone by either an urethane bond (PVA-TMR) or an amide bond (PVA-NIR), which has been shown to be very stable in vivo and in vitro.28,35

The labeling of the PVA backbone with TMR dye was conducted as described previously.25 For NIR labeling, it was necessary to synthesize different intermediates as shown in Figure 1. The main synthesis steps were the tosylation of PVA, followed by the azidation of tosylated PVA, thenamination, and finally a conjugation reaction with the fluorescence dye. Both azidation and amination reactions were quantitative. The degree of functionalization of the hydroxyl groups to amine groups was 0.68 mol% (n = 30 in Figure 1). Between one and four of the amine groups per chain were then conjugated with the NIR dye. The overall degree of functionalization of the hydroxyl groups with NIR dye was as low as 0.02–0.09 mol% to preserve the properties of PVA.

**In Vitro Filtration Experiments**. Centrifugal and pressure filtration experiments with modified PES membranes (Omega) were conducted to study the urinary excretion mechanism of high-molar mass PVA (195 kDa). The polymer, with an assumed spherical structure in water because of favorable PVA–water interactions, had a hydrodynamic radius \( R_h \) of \( \sim 13 \) nm.22 Two membrane pore sizes (NMMC of 30 and 50 kDa), much smaller than the molecular size of PVA molecules, were used to simulate the process of renal filtration in vitro. To minimize surface binding and gelation effects, which can affect centrifugal filtration, low-concentration PVA solutions (<1 mg/mL) were used. To simulate conditions closer to physiological ones, the filtration experiment with 30 kDa membranes was repeated with sodium chloride- and urea-containing solutions. All aqueous polymer solutions were generally filtered at room temperature. Possible temperature dependencies were furthermore investigated at 37 °C with an aqueous PVA solution (50 kDa membrane). The results of centrifugal (a) and pressure (b and c) filtrations of different PVA or dye-labeled PVA solutions at room temperature are shown in Figure 2.

Filtrate concentrations (Figure 2a,b) demonstrated that there is a considerable permeation of PVA molecules through membranes with a nominal pore size of \( \sim 5 \) nm (NMMC of 50 kDa) and also of <4 nm (NMMC of 30 kDa). On average, filtrate concentrations between 20 and 30% of the initial, low concentrations of PVA solutions (0.1–0.2 mg/mL) were found. As expected, an increase in the filtrate concentration and a decrease in the filtration rate were found with increasing initial PVA concentrations (Figure 2b).

We also noticed a decrease in the filtration rate over time (because of the increasing viscosity of the retentate), but no time dependence of the permeation rate (micrograms per minute per square centimeter), calculated as a product of the filtration rate and filtrate concentration, was found (results not shown). Permeation rates calculated for PVA-NIR and PVA filtration experiments (\( \sim 0.1 \) mg/mL) were 0.3 μg mL\(^{-1}\) cm\(^{-2}\) on the 30 kDa membrane (centrifugal filtration, Figure 2a) and 4.3 μg mL\(^{-1}\) cm\(^{-2}\) on the 50 kDa membrane (pressure filtration, Figure 2b), respectively. These results showed clearly the correlation of permeation and pore sizes. Repeating the PVA filtration experiment (50 kDa membrane) at 37 °C resulted in a permeation rate that was twice as high (8.6 μg mL\(^{-1}\) cm\(^{-2}\)), indicating a strong influence of temperature on the permeation mechanism.

The influence of salt and urea on the permeation of PVA is shown in Figure 2a. Higher ionic concentrations and conditions closer to physiological ones facilitated the permeation. The cleavage of hydrogen bonds as a normal effect of salt and urea seemed to allow changes in the molecular shape of PVA and to enhance the flexibility of the chains. This is in accordance with size and conformation studies of Ficoll.37 These also showed that...
the conformation of the globular polymer changed in a physiological saline solution to more extended coiled structures, which facilitates their movement through a filtration membrane. The molar mass distributions of the filtrate (\(M_0 = 143.2 \text{ kDa}\)) related to the retentate (\(M_0 = 187.7 \text{ kDa}\)) and to a freshly prepared aqueous PVA solution (\(M_0 = 174.8 \text{ kDa}\)) are shown in Figure 2c. Compared to that of the filtration experiments, the order of elution is reversed for GPC experiments. Therefore, the shifting of the filtrate curve to longer retention times (volume inside the GPC column) corresponds to an accumulation of lower-molar mass fractions in the filtrate. However, the shape of the filtrate curve at shorter retention times further indicates that PVA with higher molar masses can also cross the pores. The curves of the retentate and fresh PVA solution virtually overlap. This is due to the fact that the samples (filtrate and retentate) were taken after filtration for only \(\sim 50 \text{ min}\), corresponding to 60 mL of filtrate. At that time, the amount of higher-molar mass PVA in the retentate is still large. If the experiment would be performed in a continuous mode over several days, much larger amounts of high-molar mass PVA would be present in the filtrate.

In summary, these experiments proved that high-molar mass PVA molecules are able to permeate small membrane pores and explained previous in vivo findings about renal clearance of high-molar mass PVA molecules. The time independence of permeation rates together with GPC results as well as the enhanced permeation from salt and urea solutions (at room and body temperatures) suggests that changes in the molecular shape of the flexible molecules are responsible for the movement of the PVA molecules through small membrane pores. The renal filter process seemed to follow a kind of reptation mechanism, snake-like motion of the polymer chains through the pores that is enhanced under physiological conditions. A pore can be described as a virtual tube. The polymer is limited to a one-dimensional motion inside the tube (local reptation) followed by a slow diffusive creeping motion out of the tube (reptation).38

**Confocal Imaging of a Filtration Experiment.** The filtration (fluorescence-labeled PVA (PVA-TMR) was visualized in a filtration unit with a 50 kΩ Omega filter disk (nominal pore diameter of \(\sim 5 \text{ nm}\)) by confocal \(x/z\)-scanning. The fine porous surface of the anisotropically composed membrane (Figure 3a), responsible for size selection, was observed as a white, strong reflecting layer (Figure 3b). The “inner structure” with a lower optical density appeared dark. The air above the membrane surface is colored black because of the absence of optical density and fluorescence (Figure 3b). The same section was further observed by CLSM after addition of a PVA-TMR solution on top of the filter (Figure 3c,d). The detected emissions (after 5 and 10 min) of PVA-TMR were measured and are colored green in the two-channel overlay images (Figure 3c,d). The images clearly show the accumulation of fluorescent material below the surface, inside the membrane. Furthermore, we succeeded in imaging single penetrations of TMR-labeled PVA into the fine porous surface at certain times (marked in Figure 3d by an arrow). Because the recording procedure consists of single scans with average frame accumulations of 10 times, each image represents a period of diffusion of 16 s. CLSM cannot visualize single PVA-TMR molecules or events inside pores with a 5 nm diameter. In the image, incident simultaneously occurring diffusion events at neighboring pores of the observed \(x/z\)-section were imaged. These confocal images convincingly proved the ability of 195 kDa PVA molecules to pass small membrane pores.

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**Noninvasive PVA-TMR in Vivo Experiments.** In vivo measurements were performed using two dyes, the rhodamine dye TMR and the NIR dye Alexa Fluor 750, which had different fluorescence emission maxima (cf. Figure 5f) and varied fluorescence quantum yields. This allowed in the case of PVA-TMR, 20-fold lower exposure times, which strongly reduced the measurement recording time. Therefore, PVA-TMR imaging files (cubes) could be acquired in 2 nm steps (PVA-NIR ones in 10 nm steps) without blurring. This increased the amount of multispectral cube information. The sharp and characteristic emission maxima of PVA-TMR (cf. Figure 5f) allowed us to distinguish between tissues that were in different depths and surroundings in the body. Thus, fluorescence from accumulated PVA-TMR molecules in the abdominal fat tissue could be separated from sc PVA-TMR signals (cf. Figure 5f). Because of high photon absorbance (mainly caused by hemoglobin) of dyes with fluorescence emissions in the visible wavelength range (350–700 nm) such as TMR, they are unsuitable for analysis in underlying body tissues. Also, the accumulation of PVA-TMR in organs that are supplied with a high level of blood (kidney, liver, and spleen) would not been detectable. Therefore, dyes with fluorescence emissions in the NIR region (700–900 nm) are required.34,39,40 The body absorption in the NIR bandwidth was at least 1 order of magnitude lower than in the VIS part. This weakened the influence of light absorption in the NIR region and resulted in dominating elastic scattering effects of emitted photons. Via this phenomenon, NIR light transport in tissues can be modeled as an isotropic diffusing process in which an exact localization of the emitted fluorescence light beyond the surface becomes challenging.41 Obtained cubes have less spectral information (10 nm acquiring steps) and “smoother” emission spectra (cf. Figure 5f). Diversified determinations of signals from different body areas as described for PVA-TMR were not possible. Because of the lack of background fluorescence within
Supplemental material

(a) Investigation of the in vivo fate of a water soluble polymer

Figure 4. Unmixed, time-dependent fluorescence grayscale and corresponding intensity-weighted incremental jet color images of the sc (a and b) and ip PVA-TMR spectra (c–h) (cp. panel f). The black arrows mark an artifact caused by reflections of the xiphoid cartilage (b) and PVA accumulation in the abdominal fat depot (e and g). The white arrow (c) points to visible blood vessels.

The NIR window, fluorescence emission signals can clearly be separated from nonspecific body backgrounds. Combination experiments with both dyes, TMR and Alexa Fluor 750, were suitable for obtaining detailed overviews of the behavior of PVA in the body.

Grayscale images of detected PVA-TMR emission signals of a female mouse 1 day after injection are shown in panels a and c of Figure 4. Pixels that were allocated to the background were set to black. Pixels that could be 100% assigned to in vivo PVA-TMR (sc) (Figure 4a) or PVA-TMR (ip) signals (Figure 4c) were displayed white. Between those colors, there was an incremental gradation according to the respective intensity. The grayscale image of PVA-TMR (sc) (a) shows the homogeneous distributed polymer within and directly under the skin. Specific accumulations were indeterminable in the grayscale image. The corresponding jet color image (Figure 4b) allowed us to visualize the intensity variations within the mouse. Two areas with higher intensities indicating polymer accumulation could be identified. An enrichment between the head and the abdomen (marked by an arrow) was assigned ex vivo to the xiphoid cartilage.42 The high intensity in this area was the result of fluorescence reflections. Excitation light was strongly reflected by the xiphoid cartilage. Subcutaneously accumulated PVA-TMR polymers were excited by larger amounts of fluorescent light. Thus, the amount of emitted light increased and was furthermore also reflected by the xiphoid cartilage. Both effects influenced the intensity value in the intensity image (Figure 4b) and resulted in an apparent accumulation. Enriched fluorescent PVA-TMR molecules in the abdominal fat area are visible in the second, high-intensity area in the lower part of Figure 4b.

One day after application, the PVA-TMR (ip) signal revealed the blood vessels under the skin (marked by the white arrow in Figure 4c) due to still circulating PVA-TMR polymer chains. This confirms previous PVA results and is in accordance with blood half-life times of high-molar mass PEG molecules.22,43 Brighter parts were detected around the bladder and could be clarified in the corresponding jet color image (Figure 4d). This illustrates accumulated PVA-TMR molecules in the lower abdominal area. The same results were obtained within all female mice. Ex vivo studies confirmed the accumulation and identified them as enrichment in the abdominal fat tissue. As described previously,34 PVA was distributed from the ip injection site to the whole body either by bloodstream or by diffusion processes. Physically distributed PVA accumulated at high levels in abdominal-, kidney-, and subcutaneous-fat tissues. Once in fat tissue, incorporated PVA-TMR polymer chains were only slowly released. The PVA accumulation characteristic of the abdominal fat tissue over time is illustrated in panels e and f of Figure 4. A slow decrease in fluorescence intensity in the abdomen area is visible. Similar observations, but in a differing accumulation pattern, were made in biodistribution studies with male mice (Figure 4g, h). Ex vivo experiments confirmed the accumulation of PVA-TMR in fat also in male mice. Differences in the fluorescence allocation between male and female mice (marked by black arrows) resulted from varying fat depots (in female mice located mainly in the abdominal and in male mice in the thigh areas of the legs).

Total and maximal fluorescence intensities were calculated with the Maestro software. Therefore, the underlying data of the unmixed grayscale images of the PVA-TMR (sc) and PVA-TMR (ip) signals (Figure 4a, c) were analyzed. Calculated exposure time-weighted total and maximal fluorescence intensities are displayed in Figure 5a–d. The total signal (Figure 5a), as a sum of all PVA-TMR fluorescent pixels, increased up to day 4 and decreased afterward constantly over more than 180 days. This confirmed the expected distribution of PVA in the body. After injection, the PVA-TMR was transported via the bloodstream and by diffusion from the injection site through the body, causing the fluorescent area to be enlarged. The first increase in the magnitude of the total intensity signal was not observed in the normalized maximal intensity graph (Figure 5b). After injection, high PVA-TMR concentrations were located in the abdomen and instantly under the abdominal skin. From there PVA-TMR was slowly eliminated. Therefore, the maximal intensity remained constant for a few days and decreased afterward. An intensity value of 0.35 after 180 days indicated that there was still a small amount of accumulated PVA-TMR molecules.

PVA-TMR (ip) graphs (Figure 4c, d) had different profiles. The total intensity (panel c) was doubled during the first days,
because of the depot-forming enrichment in the fat tissue. Afterward, the magnitude of the signal decreased continuously, similar to that of the PVA-TMR (sc) signal, but faster. Comparable characteristics were found for the maximal signal intensities (panel d). After injection, PVA-TMR was physically distributed by diffusion processes throughout the body, which caused it to accumulate in fat tissues. On the basis of the poor blood supply in the fat, accumulated PVA was only slowly eliminated from the borders of the fat tissue (illustrated in Figure 6). Thus, a concentration profile existed from the center to the border of the fat tissues. Because of these restrictive eliminations, fluorescence from inner fat parts mainly influenced the maximal intensity whereas the whole fat area was represented by total intensity results. Therefore, the total intensity decreased faster than the maximal one.

**Noninvasive PVA-NIR in Vivo Experiments.** Weakened absorption effects of light emitted from NIR dyes result in a domination of the elastic scattering effects during excitation and emission processes. In the case of NIR, multiple scattering is the main mechanism for light propagation. Light transport in tissue
can be modeled as a simple isotropic diffusing process. Resulting images, shown in Figure 7a, are smoother and have less detailed information. The detection of single blood vessels, as shown with PVA-TMR, was not possible. The compare imaging function used for Figure 7a allowed correlations among all images to the comprehensive maximal intensity. One day after injection, PVA-NIR was distributed throughout the whole body. The highest levels of accumulation of dye were visible in the abdomen area (Figure 7a). This underlined the accumulation of PVA in the abdominal fat tissue discussed before. Increased intensities in the upper part (marked by top arrow) were again assigned to reflections by the xiphoid cartilage. The reduced intensity compared to PVA-TMR measurements (Figure 4b) was caused by better penetration depths of NIR light with consequently weakened reflection effects. The left kidney in the figure was marked by the second, black arrow. Both kidneys were detectable in vivo from the back side for approximately 3 days (images not shown). Accumulated fluorescence in the liver or spleen was not found within all measured mice in vivo. Over time, the whole body intensity decreased continuously (Figure 7a). Sixty-three days after polymer injection, only a homogeneously distributed, weak fluorescence signal was still detectable in vivo. Differences from the PVA-TMR image (cf. Figure 4) were attributed to the compare imaging function. Calculated total intensities based on in vivo PVA-NIR body images are shown in Figure 5e. The total intensity decrease of the received graph was influenced by the elimination of the polymer from sc and fat tissue accumulations and was intermediate between the PVA-TMT (sc) and PVA-TMT (ip) curves (Figure 5a,c). PVA-NIR Ex Vivo Experiments. Representative ex vivo images of different organs that are known for elimination (kidneys, liver, and spleen) and of skin and fat tissues (abdominal and kidney fat and skin) were imaged 3 days and 3 months after injection and are shown in panels b and c of Figure 7, respectively. Three days after injection, highest intensities were found in both kidneys. Only slight accumulations were detected in the liver and none in the spleen (panel b). Three months later, residual accumulation in the kidneys but none in the liver was detectable. The intensity in the kidneys also decreased to zero after 9 months (picture not shown). The images confirmed the preferential renal elimination pathway and were in agreement with earlier results. Ex vivo images of different fat and skin tissues agreed also with in vivo results. The elimination from the main accumulation compartments was followed over several months (Figure 7c). Different intensities between fat tissue from the abdomen or around the kidneys could be attributed to varied distances within the diffusion process after ip injection.

Ex Vivo Confocal Imaging. More detailed cellular ex vivo examination was conducted by confocal microscopic analysis. The image of abdominal fat tissue (Figure 7d, 6 months after PVA-TMR injection) confirmed PVA-TMR enrichment in fat tissues. Also, a simultaneous comparison with untreated abdominal fat clarified this (Figure 7e). An in vitro experiment included incorporation of untreated fat tissue for 3 min with PVA-TMR.
followed by washing and imaging (Figure 7f). This test confirmed the strong tendency of high-molar mass PVA molecules to passively accumulate in fat tissues. Even several washing steps could not elute the PVA-TMR. The image showed further that the highest fluorescence intensities were located between the fat cells. Entering the cells during this in vitro experiment is unlikely. Ex vivo images of treated mice resulted in a similar accumulation pattern (cf. Figure 7d,f).

**CONCLUSION**

The in vivo fate of high-molar mass PVA (195 kDa) was studied in detail by multispectral fluorescence imaging. For this purpose, two different dyes, TMR (a rhodamine dye) and Alexa Fluor 750 (a NIR dye), were covalently bound to the PVA backbone. Both dyes had different spectral properties, showed satisfactory stability in vivo, and allowed extensive in vivo studies of body distribution, accumulation, and elimination processes over several months. After i.p. injection into nude mice, the labeled PVA was distributed either in the bloodstream or by passive accumulation in fat tissues. Even several washing steps could not elute the PVA-TMR. The image showed further that the highest fluorescence intensities were located between the fat tissues such as abdominal fat and kidney fat and also under the skin. Ex vivo PVA-TMR and PVA-NIR fluorescence imaging and additionally PVA-TMR confocal microscopy studies confirmed such enrichment. It was found that PVA molecules accumulated between fat cells. Detailed quantitative analysis indicated a continuous, long-term release of PVA. The used dyes allowed us to study different aspects of accumulation of PVA in vivo, and release of PVA from several tissues. The in vivo stable PVA-NIR label allowed further characterization of elimination pathways. Strong fluorescence in the kidneys was detectable in vivo for up to 3 days. This confirmed previous studies in which PVA was found to be excreted within the urine. Slight accumulation of high-molar mass PVA was also detected in liver ex vivo. This could be attributed to the high level of blood supply to the liver causing the preferential transport and accumulation of labeled PVA. Finally, PVA was eliminated from the liver by feces, which is in accordance with literature. Nine months after i.p. application, PVA-NIR fluorescence in the liver was below the detection limit, which suggests a complete elimination of PVA.

To confirm the renal excretion of high-molar mass PVA (M_w = 195000 g/mol) and to study the involved mechanism in more detail, in vitro filtration experiments serving as a model for glomerular filtration were performed. Filtration results and x-ray scans by confocal imaging proved that despite their large molar mass and R_g value, PVA molecules were able to permeate through small pores of synthetic membranes with molar mass cutoffs of 30 or 50 kDa. Permeation rates increased with temperature and were time-independent. This suggests, together with GPC results, that changes in the molecular shape of the flexible molecules allowed them to pass through the small membrane pores. Enhanced permeation from salt and urea solutions indicated that this mechanism was favored under conditions that are closer to physiological ones.

The accumulation of PVA molecules in various fat tissues, observed in vivo and confirmed ex vivo, is not critical for clinical PVA usage. On the basis of this study and previous studies, no evidence indicating that PVA could accumulate permanently in body tissues or be toxic to cells was found. The results regarding the in vivo release of PVA from fat tissues attested the excellent properties of PVA for a controlled long-term release of bound drug molecules or proteins. These studies could open a new field of application for PVA as a controlled release drug carrier.

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**REFERENCES**

(a) Investigation of the in vivo fate of a water soluble polymer
In-vivo studies on intraperitoneally administrated poly(vinyl alcohol)

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Abstract: The fate of poly(vinyl alcohol) (PVA, 195,000 g/mol) was studied in rabbits and nude mice after intraperitoneal (i.p.) administration. In-vivo fluorescence imaging using nude mice allowed for studies of tetramethylrhodamine labeled PVA distribution in the body and tracking the urinary excretion. The excreted PVA was studied in detail after collecting the urine of rabbits over a time period of 28 days. The PVA was separated from the urine by dialysis and analyzed by FTIR spectroscopy, 1H-NMR spectroscopy, and size exclusion chromatography (SEC). Even after extensive dialysis, it was found that the excreted PVA showed a characteristic brownish color. The spectroscopic techniques revealed that this color was caused by the urine pigment (a metabolite of bilirubin) that could not be separated completely from the PVA. SEC showed unambiguously that the PVA with the very high molar mass had a glomerular permeability in the kidneys. Simultaneously, histological studies of the kidneys and the liver demonstrated that the tissues did not show any obvious damage. © 2009 Wiley Periodicals, Inc.

Key Words: poly(vinyl alcohol), PVA, in-vivo imaging, urinary excretion, fluorescence

INTRODUCTION
Poly(vinyl alcohol) (PVA) is widely used in the area of industrial, medical and pharmaceutical application, cosmetics, textile, and food packaging since the 1930.1 The high content of hydroxyl groups provides PVA and PVA-based materials with many properties suitable for biomedical applications (e.g. hydrophilic, biocompatible, nontoxic, non-carcinogenic, nonimmunogenic, and inert in body fluids).2–5 As a promising biomaterial, diverse research has focused on the application of PVA in biomedical and pharmaceutical fields.6–9 High mechanical strength, rubber-like elasticity, low-protein adsorption, high water content, and no adhesion to surrounding tissues make PVA gels a potential material for soft contact lenses,10,11 soft tissue replacements,12 articular cartilage,13 intervertebral disc nuclei,14 transcatheter arterial embolization agent,15 artificial skin, and vocal cord.16,17 Sponge-like PVA cryogels contain macropores providing properties desired for scaffolds for tissue engineering, for immobilizing of molecules and cells.18,19 On the basis of the semicrystalline structure of the PVA gel, it was studied extensively as a drug release system for pharmaceutical applications.20–24 Mucoadhesive and nonimmunogenic characteristics of PVA hydrogels were investigated for accelerating wound healing and preventing postoperative adhesion.25–27

Physiological responses of the administered PVA are dependent on molar mass and route of administration. Orally administered PVA is harmless.28 Subcutaneously administered PVA was studied using different molar masses of PVA.29 Pharmacokinetics and biodistribution of PVA were studied after intraperitoneal (i.p.), subcutaneous (s.c.), and intramuscular (i.m.) administration, which indicated that the absorption rate from the injection sites into the blood circulation was i.p. > i.m. > s.c.30 The absorption of i.p. administered PVA solutions follows two main pathways through the large area peritoneum to distribute in the whole body. One way is that PVA is absorbed into the peritoneal blood microcirculation and drained into the portal vein by passing through the liver to arrive in the blood circulation.31 Another way is that PVA was transported through the peritoneal lymphatic system directly into the blood circulation. The absorption rate of i.p. administered medium molar mass PVA showed no molar mass dependence.32 The blood concentration of PVA reaches a maximum with time after i.p. injection, and decreases more quickly with decreasing molar mass of PVA. The fate of PVA in the body is mainly governed by the hydrodynamic size of the macromolecule (single polymer chain or microgel) and the injection site.33 The main routes for elimination of PVA from the blood circulation seem to be the excretion via the renal glomeruli and hepatic bile ducts. Low molar mass PVA will be more rapidly excreted through the kidney into the urine.34–36 The critical cut-off molar mass of PVA for the glomerular permeability was reported to be 30,000 g/mol.37 Although the molar mass and the molecule size of PVA is above the size limitation of the glomerular filtration barrier (nonfilterable

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(a) Investigation of the in vivo fate of a water soluble polymer

if molar mass \(>80,000\) g/mol and radius of molecule \(>4.4\) nm), i.p. administrated high molar mass PVA \((M_w = 195,000\) g/mol, \(R_g \approx 13\) nm) can still be excreted through the kidney.\(^{38,39}\) High molar mass water soluble polymers take several weeks or months to be finally excreted through urine and feces. A significant accumulation of high molar mass polymers has been observed in the liver and spleen, but minor amounts of them can be deposited in the body.\(^{40-42}\) Limited information is available on PVA biodegradation mediated by cells other than microorganisms in soil and bacteria.\(^{43-45}\) Until now, PVA is usually regarded as a nonbiodegradable polymer in the body. The delay of renal excretion of high molar mass PVA is still not completely explored. Recent studies mentioned that the chemical structure of renal excreted PVA differs from pre-administered PVA.\(^{39}\) To gain a better understanding on renal excretion of high molar mass PVA, the urinary excreted PVA i.p. administered to rabbits is investigated in this study using GPC, FTIR spectroscopy, and \(^1\)H-NMR spectroscopy. The biocompatibility of PVA is studied by histopathological tests of the kidney. The fluorescence-labeled PVA using a tetramethylrhodamine-5-carbonyl azide dye (PVA-TMR) is used to follow its fate in the body of nude mice after i.p. administration. The main focus is to measure the PVA-TMR distribution from the area of injection into the peritoneum and blood stream. Finally, the labeled PVA-TMR is analyzed in the urine to prove the elimination through the kidneys.

MATERIALS AND METHODS

Materials

The PVA (Mowiol 56–98, Kuraray, Japan) had a weight average molar mass \(M_w\) of 195,000 g/mol, a polydispersity index of 2.3, and a degree of hydrolysis of 98.4 mol %. All other chemicals and solvents were used as received.

Preparation of Fluorescence Labeled PVA

The PVA was labeled with the fluorescence dye tetramethylrhodamine-5-carbonyl azide (TMR) (Invitrogen) in anhydrous dimethylsulfoxide (DMSO) at 80\(^\circ\)C for 12 h using a molar ratio PVA/TMR (2.2/1) according to the reaction scheme shown in Figure 1.

After completion of the reaction the solution was dialyzed against DMSO through a membrane with molar mass \(M_w\) of \(>4.4\)\(\times\)10\(^6\) g/mol and radius of molecule \(>120\) nm, i.e., a cut off of \(3500\) g/mol for removing unconjugated tetramethylrhodamine. Both the dialysis bag and the DMSO were changed daily until no trace of TMR in the organic solvent. Again, both the dialysis bag and the DMSO were changed daily until no trace of TMR in the aqueous solution was detected through fluorescence spectroscopy. Finally, the aqueous solution was freeze-dried to a powder, readily soluble in water at 80\(^\circ\)C. The fluorescence emission spectrum of PVA-TMR for an excitation wavelength \(\lambda_{exc} = 435\) nm shows a maximum at \(\lambda = 577.3\) nm that is not shifted compared to the emission maximum of TMR \((\lambda = 577.0\) nm), although the PVA-TMR spectrum is slightly narrower compared to the pure TMR. This indicates that the conjugation did not influence considerably the fluorescence of the original TMR.

In-Vivo Imaging and Analysis

The measurements were carried out using an in-vivo fluorescence imaging system (Maestro, Cambridge Research & Instrumentation, Woburn, MA). The distribution and accumulation of the labeled PVA was studied by measuring the fluorescence intensities of the whole body, covering especially the abdominal region, at predetermined time intervals. Therefore, a green excitation filter (503–555 nm) and a 580 nm long-pass emission filter were used. Multispectral imaging cube sets were acquired in 2 nm steps (spectral range between 500 and 800 nm) with an exposure time of 50 ms. An original diluted PVA-TMR solution was used to generate the reference fluorescence spectra for further analyses. By unmixing and further segmentation it was possible to separate the PVA-TMR signal from auto-fluorescence signals of the mice. Corresponding images were saved into a series of monochrome images. RGB (red green blue) images were generated from the original cubes by allocating the respective color to the labeled PVA spectrum.

All procedures of the in vivo experiments complied with the standards for use of animal subjects as stated in the guideline from the animal care and use committee of the Martin Luther University Halle-Wittenberg. The in vivo studies were performed in nude, female mice (SKH1-Hrhr, 25–30 g) from Charles River Lab. They were housed under controlled conditions (12 h light/dark schedule, 24\(^\circ\)C). The fluorescent PVA-TMR solution was slowly i.p. injected (5 wt %, 0.1 mL) into the middle of the abdomen. For the
measurements, an inhalation anesthesia system was used with a mixture of isofluorane/oxygen and an initial flow of 4% isofluorane (3.0 L/min oxygen) and a steady state flow of 1.8% isofluorane (1.5 L/min oxygen). To protect the mice against cooling, they were placed on temperature controlled plates which were kept at 35°C.

The urine samples were collected directly before the in vivo measurements. Therefore, the mouse was placed into an empty mouse cage for less than 2 h. In this time, the mouse urinated to the bottom of the cage. Immediately after the collection, the urine samples were diluted with acetonitrile (1:100) and measured with the fluorescence imaging system using a blue excitation filter (445–490 nm) and a 515 nm long-pass emission filter as well as the green filter set. A blue filter set was used to detect the native urine components and the green one for analyzing the PVA-TMR signal. Semiquantitative data analysis about the presence of labeled polymer in the urine was possible by combining both spectra.

Extraction and Dialysis of Polymer Obtained by Renal Excretion of Rabbits
Sterilized 20 mL of 10 wt % PVA solution was injected into the abdomen of three female albino rabbits (namely No. 3196, No. 3242, and No. 3965) by laparotomy. PVA-treated rabbits’ urine was collected and deep frozen immediately. After 28 days urine collection, all urine samples were thawed and filtered to remove any solid material (e.g. hay particles from the animal cage). The urine of 28 consecutive days (generally 1.1–1.5 L, deep brown color) was filled into a dialysis tube (MWCO 3500 g/mol, Spectra/Por3, Spectrum Laboratories, CA) and dialyzed against distilled water for 4 days with changing distilled water three times per day. Then, the dialyzed urine (light brown color) was distilled under vacuum to remove most of the water. The concentrated urine (generally 30–50 mL, deep brown color) was dialyzed against distilled water for 2 days with changing distilled water three times per day. Precipitates can be collected after pouring the concentrated urine into acetone. The precipitated polymers in acetone were filtered by glass filters. The amounts of collected polymers were about 200, 500, and 140 mg, respectively. The control sample was dialyzed and precipitated by mixing fresh PVA aqueous solution with rabbit urine at room temperature. One milliliter of 10 wt % PVA (100 mg) aqueous solution was added into 200 mL rabbit urine. The control sample then underwent the identical procedure as discussed for the collection of polymer from the rabbit urine samples.

Size Exclusion Chromatography
Molar masses of polymers were measured by SEC at ambient temperature using a Waters size exclusion chromatography (SEC) equipped with a Knauer pump. Poly(ethylene oxide) calibration standards were used to calculate the molar masses. Samples were measured in an aqueous environment. The SEC traces were normalized so that the highest peak represents 100% of detector response.

1H-NMR Spectroscopy
The molecular structure of the polymers was determined by 1H-NMR spectroscopy. 1H-NMR spectra were recorded using a Magnetic Resonance spectrometer (Gemini 2000, Varian, 400 MHz) at 20°C in DMSO-d6.

FTIR Spectroscopy
The polymer powder was pressed into KBr tablets and the spectra were recorded in the transmission mode of a FTIR spectrometer (Tensor 37, Bruker) with a resolution of 2 cm⁻¹. One thousand interferogram scans were averaged to give spectra from 400 to 4000 cm⁻¹.

Histological Test
To observe if the high molar mass PVA can produce toxicities in kidney, the histopathological changes of the kidney tissue of control and PVA-treated rabbits were examined by hematoxylin and eosin (H&E) stained slides. After 28 days of urine collection, three PVA i.p. administrated rabbits and one control rabbit (without PVA administration) were killed and autopsied and the kidneys were removed and deep frozen (at −32°C) immediately. The sections (4 mm × 10 mm × 10 mm) of tissue were sampled from deep frozen organs and fixed in neutral-buffered formaldehyde-solutions, processed through graded alcohols and xylene, and embedded in paraffin blocks. Tissue sections were cut for 2–8 μm at multiple levels and routinely stained with hematoxylin-eosin. Mounted slides were examined and photographed under a light microscope.

RESULTS

Fluorescence Imaging
It has been shown that in-vivo fluorescence imaging is a powerful tool for studying the fate of biomedical materials and anatomical changes in the body of animals. The aim of the experiments was to follow the fate of the labeled PVA after i.p. injection. Shortly after administration, the PVA-TMR solution spreads into the abdomen cavity. At this moment, the PVA-TMR is localized in the middle of the abdomen between the intestinal loops. Parts of the fluorescence signal are absorbed by the intestine which results in apparently nonfluorescent areas. The fluorescent areas show a quenched, high-intensity signal caused by the highly concentrated PVA-TMR solution. Over time the labeled PVA distributes inside the abdominal space. From there it is absorbed to the body fluids. It was possible to detect the PVA-TMR signal in the big vasculatures under the skin after 6 h. The polymer circulates with the bloodstream in the whole body. At the abdominal site the fluorescence area increases whereas the intensity of the signal decreases (i.e. there are less areas with quenched signal). The fluorescence intensity is adapted to a medium value as illustrated in Figure 2(e). It is visible that the total fluorescence area is enlarged and the color changed from dark red to yellow and blue. After 1 day, the PVA-TMR was still visible in the blood vessels. Furthermore, we detected an accumulation of the polymer in the upper abdomen space. The jet color picture illustrates the unusual V-profile of the
(a) Investigation of the in vivo fate of a water soluble polymer

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from red to yellow and blue. It is obvious that the PVA-TMR signal can be detected in the mouse urine shortly after i.p. injection. The highest fluorescence intensity was found 14 h after injection indicating that the PVA-TMR was taken up by the body fluid and circulated in the blood stream. Finally, it was eliminated through the kidneys into the urine. After 5 days the amount of renal excreted PVA-TMR decreased under the detection limit of the fluorescence imaging system. It was impossible to detect PVA-TMR in the feces at any time. Thus, we conclude that most of the labeled i.p. administrated high molar mass PVA is excreted through the kidneys.32

Characterization of Dialyzed-Precipitated Polymer

The polymers extracted from the dialyzed urine of rabbits after i.p. administration of PVA show a brown color. To characterize the excreted polymer in more detail, original PVA, control sample (PVA mixed with rabbit urine), three extracted urinary samples, and the urine pigment were investigated by SEC, FTIR spectroscopy, and 1H-NMR spectroscopy. This brown color is most likely caused by metabolites of bilirubin (urobilin)—the final degradation product of hemoglobin (Figure 4).19 The urine pigment sample as a reference is used to identify the influence on the precipitated polymers. The urinary extracted polymer sample was treated by alkaline solution (1 mol/L NaOH). Thus, the aggregates of excreted PVA with urine pigments were partially broken. This treated sample was then poured into acetone. PVA is not soluble in acetone and precipitates. The suspension is centrifuged and the supernatant liquid is collected and evaporated at 70°C. A deep brown solid urine pigment was obtained after evaporation. The dried PVA had still a brownish color.

Figure 5 shows the FTIR spectra of extracted urinary samples, the original PVA, the control sample, and the urine pigment. The IR spectra exhibit several bands characteristic of stretching and bending vibrations of O–H, C–H, C–O, and C=O groups. The significant observed IR band positions and respective functional groups are listed in Table I. The characteristic bands of pure PVA are located at 3332, 2942, 1440, 1325, 1094, 916, and 850 cm⁻¹. The broad and strong band observed at about 3300 cm⁻¹ corresponds to the O–H stretching vibration. A weak band at 1325 cm⁻¹ has been assigned to the combination frequency of C–H and O–H groups. The strong band at 1094 cm⁻¹ is attributed to the stretching mode of C–O of PVA. The band at 916 cm⁻¹ is assigned as the stretching mode C=O depending in the tacticity of PVA. The broad band at 2942 cm⁻¹ (3000–2800 cm⁻¹) is assigned to the overlapping of asymmetric and symmetric C–H stretching of CH₃ and CH₂ groups. Other bands appear at 1440 and 850 cm⁻¹ that are related to bending modes of the CH₂ group. Most of the characteristic bands of PVA can also be observed in the IR spectra of urinary extracted polymers. Additional bands at 1649, 1542, 1406, and 1237 cm⁻¹ can be observed in the IR spectra of the extracted urinary samples. These intense bands can be related to the urine compounds by comparing

![Figure 4. Chemical structure of urobilin. (Bilirubin reduction in the gut leads to urobilinogen which is oxidized to urobilin by intestinal bacteria. Urobilin is absorbed into the blood stream and is finally excreted in urine.)](image)

![Figure 5. FTIR spectra of original PVA, control sample (PVA mixed with rabbit urine and dried), and dialyzed-precipitated polymer of the rabbit urine samples (PVA i.p. administrated rabbits No. 3196, No. 3965, and No. 3242) and rabbit urine pigment.](image)

![Table 1. Infrared Absorption Frequencies and Vibrational Modes Related to Poly(vinyl alcohol) and Dialyzed-Precipitated Polymer of the Rabbit Urine Samples](table)
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To further identify the molar mass of urinary excreted polymer, attempts were made to break the interactions between the polymer and the urine pigments by acid or alkaline treatment. 1 mol/L NaOH or HCl are added to the acidic environment, urine pigment interacting with PVA molecules is partly released from the polymer conjugation. Alkaline and acid treatments show a similar influence on the breaking of the aggregates of excreted polymer and urine pigments. NaOH of 1 mol/L is then chosen to hydrolyze all three extracted urinary samples. SEC traces exhibit three obvious peaks in base-treated extracted urinary samples indicate that three main compounds are contained in the urinary extracted sample (Figure 8). The peaks at $V_R$ of 12 mL represent the released urine pigment after basic treatment. The other two peaks distribute broadly at $V_R$ of 6 and 10 mL, respectively. The nonuniform distribution of these two peaks indicates the existence of different polymer-urine pigment aggregates in the urinary excreted samples. The shape and the different shifts of $V_R$ of these peaks make it difficult to characterize the excreted PVA exactly with respect to the molar mass. The peaks that appeared in the range of 9–11 mL can also be observed in SEC traces of the control sample and it must be related to urine pigment-polymer aggregates. The peak at intermediate retention volumes around 9.5 mL might be caused by aggregates that are only partially cleaned under the experimental conditions selected.

**Histological Tests**

The biocompatibility of PVA was investigated by histopathological tests of the kidneys which were excised from the PVA i.p. administrated rabbits. H&E (hematoxylin and eosin) stain is a popular staining method in histology. The staining method involves application of the basic dye hematoxylin, which colors basophilic structures with blue-purple hue, and alcohol-based acidic eosin, which colors eosinophilic structures bright pink. The basophilic structures are usually the ones containing nucleic acids, such as the ribosomes and the chromatin-rich cell nucleus, and the cytoplasmatic regions rich in RNA. The eosinophilic structures are generally composed of intracellular or extracellular protein. Most of the cytoplasm is eosinophilic.

The kidney is the most important organ for the urinary excretion. Histological testing is an important method to evaluate the biocompatibility of biomaterials. In this study, the appearance of nephrotoxicity, PVA accumulation or deposition in the kidney. Similar conclusions can be drawn from the medulla section of the rabbit kidney (Figure 10). There is not any evident damage or degeneration of the tissue.

**DISCUSSION**

The urinary extracted polymer from PVA i.p. administrated rabbits shows the main spectral features of pure PVA in the investigations of FTIR and $^1$H-NMR spectroscopy. The characteristic signals represent PVA that can be detected in extracted urinary samples. The SEC traces indicate that strong aggregation occurred between the extracted PVA and urine pigments. The aggregation can partially be broken under alkaline or acid condition. The SEC traces of alkaline-treated extracted urinary samples exhibit multipeak distributions. A characteristic peak occurs at shorter retention times. Control experiments using PVA and rabbit urine mixtures show a similar peak, which is probably caused by aggregates formed due to strong physical interactions between PVA and urobilin. All the results mentioned earlier show that extracted urinary samples exhibit some obvious differences from the original PVA. They are caused by the urine compounds that could not be separated from the collected PVA (e.g. urobilin). This can be clearly seen in FTIR and $^1$H-NMR measurements.

The kidney as a main excretion gateway has selection criteria on the molecule size and molar mass of filtered substances. Molecules with $R_h < 1.8$ nm (molar mass $<$ ca. 10,000 g/mol) can be filtered through glomerular membrane without any hindrance. Molecules with 1.8 nm $< R_h < 4$ nm are only partially filterable. Molecules with $R_h > 4.4$ nm (molar mass $>$ 80,000 g/mol, e.g. globulin) usually cannot be filtered. However, additional parameters (charge, shape, and flexibility) are also contributing to the renal filtration efficiency. For example, negative charged substances have lower filtration coefficients when compared with neutral molecules with the same radius. It is also known that linear, flexible molecules of the same molar mass are more easily filtered compared to molecules with a spherical shape. The reported critical cut-off of PVA in renal filtration is 30,000 g/mol. The molar mass of PVA applied in this study is 195,000 g/mol. Both molar mass and molecule size ($R_h \sim 13$ nm) of PVA-195k are far above the limits of glomerular filtration. I.p. administered PVA-195k can be excreted gradually through kidney for a long time without damaging renal glomeruli as demonstrated in this study. It seems possible that PVA shows some kind of reptation allowing for passing the renal filter.

**CONCLUSIONS**

The urinary excretion of i.p. administrated PVA having a tetramethylrhodamine label can be studied by fluorescence imaging. The amount of excreted PVA decreases over 5 days until the detection limit for this method is reached. With extensive investigations on urinary extracted polymers after intraperitoneal administering of high molar mass PVA (195,000 g/mol) using FTIR spectroscopy, $^1$H-NMR spectroscopy, and SEC, it is confirmed that the brownish urinary...
extracted sample contains mainly PVA. The results of FTIR spectroscopy and \(^1\)H-NMR spectroscopy are in good agreement with each other. FTIR spectroscopy, \(^1\)H-NMR spectroscopy, and SEC traces of extracted urinary samples show some differences from the original PVA. The changes caused by the urine pigments are identified by the spectroscopic results of the control sample and the urine pigment itself. In histological tests, the appearance of nephrotoxicity cannot be observed in the histological sections of PVA-195k-treated rabbits by comparing with the control rabbits (not treated with PVA).

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REFERENCES
Supplemental material

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Tracking the In Vivo Fate of High Molar Mass Poly(vinyl alcohol) Using Multispectral Fluorescence In Vivo Imaging

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Introduction
Poly(vinyl alcohol) (PVA) is a nonionic, water-soluble, and biocompatible polymer (1). In the pharmaceutical industry, it is widely used for biomedical applications, such as contact lenses and scaffolds for wound healing and tissue regeneration. PVA hydrogels are also extensively studied for the controlled release of therapeutic molecules. It has also been reported that PVA membranes can be used for adhesion prevention of postsurgical abdominal adhesions (2). Adhesions are internal scars that develop after trauma and involve the injured tissue and peritoneum. An ideal barrier would be a gel or liquid solution that can be injected to the place of interest. Following peritoneal healing, it should be reabsorbed and eliminated. PVA (125,000 g/mol) elimination studies in rabbits showed that PVA passes the kidneys despite its high molar mass (3).

The question is whether the elimination results can be assigned also to a high molecular weight (195,000 g/mol) PVA. The noninvasive method of optical imaging was used to measure how long the PVA is localized at the area of injection to follow possible accumulation in the body and examine whether PVA is still eliminated through the kidneys. PVA was labeled with the fluorescence dye tetramethyl-rhodamine-5-carbonyl azide (TMR) (from Invitrogen) in anhydrous dimethylsulfoxide at 80°C. The dialyzed PVA-TMR was dissolved in water (5%, wt/wt) (Figure 1).

Methods
In vivo imaging was performed with the Maestro™ in vivo imaging system (CRi, Woburn, MA). Distribution and accumulation were studied by measuring the whole body from the abdominal side at predetermined time intervals using a green filter set (580-nm long-pass emission filter). Multispectral imaging cube sets were acquired. By unmixing and further segmentation, it was possible to separate the PVA-TMR signal from the auto fluorescence signals of the mice. The total signal, as the sum of all pixel values from the extracted PVA-TMR signal, and the maximum pixel values were then calculated.

Results
Using the Maestro™ software from CRi it was possible to separate the in vivo fluorescence PVA-TMR signal from the background signals of the mice (Figure 2). After injection of the PVA-TMR dispersions, high concentrations of labeled polymer could be detected in the area of the abdomen. From there it was distributed via the body liquids throughout the whole body (Figure 3). The circulated polymer was accumulated in the area under the skin but also in the region of the fat pad. Using the Maestro™ software it was possible to separate the accumulated PVA-TMR signals between the skin and other parts like the fat pad that were analyzed after masking the skin signal.

The total fluorescence intensities from the abdomen site of the body increased to a maximum within the first week, which was the result of the distribution throughout the whole body. After this time, the PVA-TMR was continuously released and mainly eliminated through the kidneys (Figure 4). Surprisingly, we detected obvious differences between male and female mice in

Figure 1. Chemical structure of TMR-labeled poly(vinyl alcohol).

Figure 2. Normalized emission fluorescence spectra; blue: in vivo extracted PVA-TMR spectra; black: background signal of an untreated mouse; red: manually computed PVA-TMR spectra.

Figure 3. Unmixed images of one nude, female mouse 24 hr after i.p. injection. The incremental jet color images represent the threshold fluorescence PVA-TMR signal. The red color indicates areas of detected PVA-TMR signal.

Figure 4. Total fluorescence intensities from the abdomen site of the body increased to a maximum within the first week, which was the result of the distribution throughout the whole body. After this time, the PVA-TMR was continuously released and mainly eliminated through the kidneys (Figure 4). Surprisingly, we detected obvious differences between male and female mice in

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How Stealthy are PEG-PLA Nanoparticles? An NIR In Vivo Study Combined with Detailed Size Measurements

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ABSTRACT

Purpose Detailed in vivo and ex vivo analysis of nanoparticle distribution, accumulation and elimination processes were combined with comprehensive particle size characterizations.

Methods The in vivo fate of near infrared (NIR) nanoparticles in nude mice was carried out using the Maestro™ in vivo fluorescence imaging system. Asymmetrical field flow field fractionation (AF4) coupled with multi-angle laser light scattering (MALLS), photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM) were employed for detailed in vitro characterization.

Results PEG-PLA block polymers were synthesized and used for the production of defined, stable, nontoxic nanoparticles. Nanoparticle analysis revealed narrow size distribution; AF4/MALLS permitted further accurate size evaluation. Multispectral fluorescence imaging made it possible to follow the in vivo fate non-invasively even in deep tissues over several days. Detailed fluorescence ex vivo imaging studies were performed and allowed to establish a calculation method to compare nanoparticle batches with varying fluorescence intensities.

Conclusion We combined narrow-size distributed nanoparticle batches with detailed in vitro characterization and the understanding of their in vivo fate using fluorescence imaging, confirming the wide possibilities of the non-invasive technique and presenting the basis to evaluate future size-dependent passive tumor accumulation studies.

KEY WORDS AF4 • fluorescence imaging • in vivo imaging • nanoparticle • PEG-PLA

ABBREVIATIONS

AF4 asymmetrical field flow field fractionation
CHO Chinese hamster ovary
DiR 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide
EMEM Eagle’s minimum essential medium
EPR enhanced permeability and retention
FBS fetal bovine serum
MALLS multi-angle laser light scattering
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIR near infrared
NR Nile red
PBS phosphate-buffered saline
PCS photon correlation spectroscopy
PDI polydispersity indices
QD quantum dots
RES reticuloendothelial system

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INTRODUCTION

The development of biodegradable nanoparticles as drug delivery vehicles for therapeutic agents is one of the main promises for future cancer therapies. The major aim is to produce nanoparticles which preferentially accumulate at the tumor site. Loaded with anticancer drugs, they can improve cancer therapy and simultaneously reduce the harmful nonspecific side effects of chemotherapy (1). In addition, nanoparticles loaded with contrast agents may provide very efficient cancer diagnostics by in vivo imaging. Two main tumor targeting strategies have generally been explored: First, active targeting by conjugating nanocarriers with molecules that can bind to tumor-specific antigens or receptors on the cancer cells (2), and second, the more commonly used passive targeting strategy. The first marketed products were introduced more than 10 years ago as liposomes or polymer-protein conjugates (2). The passive accumulation of nanoparticles is based on the enhanced permeability and retention (EPR) effect of tumor tissues (3). Several studies have shown that particle size plays a key role in accumulation effectiveness. Experiments using liposomes have demonstrated that the upper size limit for extravasations into tumors is about 400 nm (4). Others showed that smaller particles with sizes below 200 nm are even more effective (5–7). However, particles smaller than 20–30 nm are eliminated faster by renal excretion (8). It was also noticed that liposomes, which are smaller than 70 nm, were more rapidly cleared from the circulation than larger (between 70 and 300 nm) ones (9, 10). Therefore, the optimal size of nanoparticles for cancer treatment should be between about 70 and 200 nm (11). Accurate knowledge of the particle size, size distributions, and particle morphology is a key requirement for in vivo applications of newly developed nanoparticles, although this is often neglected. Gaumet et al. (12) characterized size-dependent accumulation studies of nanoparticles within the body and comprehensively researched further by additional ex vivo experiments. However, fluorescence imaging with different nanoparticle batches (differing in sizes and in polymer composition) may pose constraints, such as varying fluorescence intensities. Due to diversity in dye loading and dye allocation in the particles, direct comparison of the results and particularly quantification in tissues obtained with different nanoparticle batches proved challenging. In this work a new calculation approach is discussed as a basis for future evaluations of size-dependent tumor accumulation studies with different nanoparticle batches. Beyond that, exact size characterization is an important issue, as mentioned above. Particle sizing at the nanoscale is, however, by no means a trivial task. The several methods are based on different measurement principles and the potential existence of various particle species as well as heterogeneous size distribution. These are only some of the challenges in size determination of colloidal formulations. In the present study, the combination of PCS, AF4 coupled with MALLS and TEM provided comprehensive information about the size and morphology of the nanoparticles as a basis for drawing meaningful conclusions about the in vivo fate of nanoparticles.

MATERIALS AND METHODS

Materials

3,6-Dimethyl-1,4-dioxan (D,L-lactide), poly(ethylene glycol) monomethyl ether (mPEG2000; MW = 2000 Da), stannous 2-ethylhexanoate (>95%), phosphotungstic acid (reagent grade), nile red (NR), Eagle’s minimum essential medium (EMEM), nutrient mixture HAM’s F-12, sodium dodecyl sulphate and sorbitol were obtained from Sigma Aldrich, Germany. PLGA nanoparticles were synthesized as previously described (13). Two main tumor targeting strategies have generally been explored: First, active targeting by conjugating nanocarriers with molecules that can bind to tumor-specific antigens or receptors on the cancer cells (2), and second, the more commonly used passive targeting strategy. The first marketed products were introduced more than 10 years ago as liposomes or polymer-protein conjugates (2). The passive accumulation of nanoparticles is based on the enhanced permeability and retention (EPR) effect of tumor tissues (3).

In this study, detailed non-invasive in vivo fluorescence imaging experiments were combined with fundamental in vitro tests to allow meaningful data interpretation and to gain information about the in vivo fate of PEG-PLA nanoparticles. Multispectral in vivo fluorescence imaging has already been used for fluorescence tissue imaging with nanocarriers like quantum dots (QD) (15–17). Using NIR QD, it could be shown that fluorescence imaging is possible even in deep tissues (18). Nevertheless, QD are criticized due to the high production costs and the potential in vivo toxicity depending on their surface properties (19). The incorporation of the very lipophilic NIR carbocyanine dye DiR into PEG-PLA nanoparticles combined the advantages of NIR light with low toxicity risks for the animals. Fluorescence imaging thus allowed the study of distribution, accumulation and elimination processes of the nanoparticles over several days non-invasively. The distribution of the nanoparticles within the body was comprehensively researched further by additional ex vivo experiments. However, fluorescence imaging with different nanoparticle batches (differing in sizes and in polymer composition) may pose constraints, such as varying fluorescence intensities. Due to diversity in dye loading and dye allocation in the particles, direct comparison of the results and particularly quantification in tissues obtained with different nanoparticle batches proved challenging. In this work a new calculation approach is discussed as a basis for future evaluations of size-dependent tumor accumulation studies with different nanoparticle batches. Beyond that, exact size characterization is an important issue, as mentioned above. Particle sizing at the nanoscale is, however, by no means a trivial task. The several methods are based on different measurement principles and the potential existence of various particle species as well as heterogeneous size distribution. These are only some of the challenges in size determination of colloidal formulations. In the present study, the combination of PCS, AF4 coupled with MALLS and TEM provided comprehensive information about the size and morphology of the nanoparticles as a basis for drawing meaningful conclusions about the in vivo fate of nanoparticles.
Synthesis and Characterization of PEG-PLA Polymers

Biodegradable diblock copolymers PEG2PLA20 and PEG2PLA40 were synthesized following a previously established method (20). The numbers refer to the molecular weight (kDa) of the respective polymer block. The PLA part was attached to the mPEG part by a ring-opening polymerization of 3,6-dimethyl-1,4-dioxan (D,L-dilactide) using stannous 2-ethyl hexanoate as catalyst. First of all, any trace amounts of water were removed from the educts. Therefore, about 2 g of the mPEG2000 were dissolved in 100 mL toluene in a three-neck round-bottom flask and heated; the reflux of 50 mL toluene was distilled off using a water separator. D,L-dilactide and 100 mL toluene (20 g and 40 g, respectively) were added, and again 50 mL of the solvent was distilled off. The final volume of toluene thus did not exceed 100 mL. After addition of 500 μL glacial acetic acid and about 400 mg of the catalyst, the mixture was refluxed for at least 8 h under nitrogen atmosphere. Afterwards, the toluene was removed by distillation with 200 mL methylene chloride and acetone using a rotary evaporator. The obtained viscous polymer was dissolved in acetone and precipitated by dropping into water at 4°C to remove residual catalyst and water-soluble by-products. The precipitate was separated, frozen at −80°C and freeze-dried. Afterwards, the dried polymer was stored under vacuum. The molecular weight of the synthesized polymers was determined by 1H-NMR and GPC (data not shown).

Preparation of Polymer Nanoparticles

Unloaded (batch A, B and C) and loaded (DiR, batch D and NR, batch E) polymeric nanoparticles were prepared by nanoprecipitation (21). Preparation conditions, like polymer concentration, temperature, impact of volumes (polymer phase and external phase) and the influence of stabilizer, were studied preliminarily (22). For nanoparticle preparation, the respective polymer (2.5, 5 or 10 mg) and—if applicable—the fluorescence dye were dissolved in 5 mL acetone (ep. Table I). This organic solution was drop by drop (2 mL per minute) added to 40 mL of an aqueous solvent was distilled off. The particles were collected by centrifugation (12,500 g; 30 min; 10°C) using an Avanti JE high-speed centrifuge (Beckman Coulter, Germany) and washed with filtrated distilled water. The particles were re-dispersed in bi-distilled water preserved with 0.5% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Appli-Chem GmbH, Darmstadt, Germany. All other substances and solvents were used as received.

Table I Nanoparticle Compositions and Particle Sizes of Different Nanoparticle Batches Measured Directly After Redispersion

<table>
<thead>
<tr>
<th>Batch</th>
<th>Polymer  a)</th>
<th>A PLGA</th>
<th>B PEG2PLA20</th>
<th>C PEG2PLA40</th>
<th>D PEG2PLA40-DiR</th>
<th>E PEG2PLA40-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dye loading b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5%</td>
<td>0.6%</td>
</tr>
<tr>
<td></td>
<td>Polymer concentration c)</td>
<td>1.0%</td>
<td>1.0%</td>
<td>1.0%</td>
<td>2.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td></td>
<td>PCS - z-average in nm (PDI)</td>
<td>339 (0.52)</td>
<td>113 (0.09)</td>
<td>104 (0.08)</td>
<td>166 (0.13)</td>
<td>103 (0.08)</td>
</tr>
<tr>
<td></td>
<td>TEM (nm)</td>
<td>128 ± 13</td>
<td>82 ± 15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MALLS D10 (nm)</td>
<td>127 ± 10.4</td>
<td>66 ± 0.9</td>
<td>62 ± 0.1</td>
<td>48 ± 9.8</td>
<td>59 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>MALLS D50 (nm)</td>
<td>222 ± 9.0</td>
<td>95 ± 0.2</td>
<td>86 ± 1.0</td>
<td>99 ± 4.0</td>
<td>81 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>MALLS D90 (nm)</td>
<td>403 ± 29.8</td>
<td>141 ± 0.6</td>
<td>132 ± 0.4</td>
<td>230 ± 3.3</td>
<td>121 ± 0.2</td>
</tr>
</tbody>
</table>

a) The numbers 2 and 20 refer to the molar mass of the polymer block (kDa).
b) Dye loading in percent related to the polymer amount.
c) Polymer concentration in percent related to the organic solvent.
d) Sample was filtered (pore size 0.8 μm) prior measurements.
Particle Size Characterization of Nanoparticles

Photon Correlation Spectroscopy (PCS)

Dynamic light scattering was measured at 25°C in the backscattering mode (173°) with a High Performance Particle Sizer (HPPS) from Malvern Instruments (Malvern, Herrenberg, Germany). Samples were diluted with purified, filtered (0.2 μm) water (nanoparticle concentration was about 0.1 mg/mL) and measured 4 times with 12–16 runs over 10 s each at fixed measurement position in the middle of the cuvette. Due to macroscopic inhomogeneities, PLGA suspensions were filtrated (pore size 0.8 μm, PALL medical, Dreieich, Germany) and measured once again. Z-average diameters and PDI were determined by the instruments cumulant analysis software (version 4.20). Results are given as average with standard deviation of the 4 measurements (*n=4*).

Asymmetrical Flow Field-Flow Fractionation (AF4)

Samples were analyzed as described earlier (23). In brief, the fractionation system (Eclipse AF4, Wyatt, Dernbach, Germany) was coupled with a multi-angle laser light scattering (MALLS) detector (DAWN EOS, Wyatt). The trapezoidal channel (length 265 mm, largest width 21 mm, height 350 μm) was equipped with a membrane of regenerated cellulose or polycethersulfone (MWCO 5 kDa, Micromod-Nadir, Wiesbaden, Germany). Bi-distilled water preserved with 0.02% sodium azide and filtered through 0.1 μm was used as carrier liquid. One hundred μL dispersion (nanoparticle concentration about 1 mg/mL) was injected during focusing (focus flow 2 mL/min), and samples were eluted with a constant detector flow of 1 mL/min and decreasing cross flow. Initially, a high cross flow gradient (cross flow decreasing from 2 to 0.5 mL/min within 3 min) was applied to assure baseline separation of the nanoparticles from the void peak followed by a decreasing cross flow (0.5–0 mL/min within 35 min) to separate the nanoparticles. Size evaluations were done by the Astra software 4.90 (Wyatt) using the particle mode and assuming compact spheres (23). Mass weighted size distributions and the characteristic diameters (D10, D50, and D90) were calculated using the binning method (23). Similarly as in PCS measurements, the dispersion of PLGA nanoparticles was filtered (pore size 0.8 μm) prior to the measurements. The separation accuracy of the AF4/MALLS system was checked by using a mixture of 50, 100, 200 and 300 nm polystyrene standard nanoparticles as described earlier (23). All samples were measured in triplicate, and results are given as average with standard deviation (*n=3*).

Transmission Electron Microscopy (TEM)

About 10 μL of diluted aqueous nanoparticle dispersions were placed on 3.05 mm formvar/carbon-coated copper grids (300 mesh) and negatively stained with phosphotungstic acid (2% in water) for 30 s. Samples were subsequently dried under vacuum and viewed in a Zeiss EM C/CR (Carl Zeiss AG, Germany) at 60 kV operating voltage. Particle size was estimated by manually measuring the diameter of 100 randomly chosen particles for each sample. The mean size was determined for each sample in triplicate (*n=3*).

In Vitro Cytotoxicity Assay

Biocompatibility was tested with hamster endothelia (Chinese hamster ovary (CHO) cells and mouse fibroblasts (L929). They were cultured in HAMs F12 and EMEM, respectively, each supplemented with 10% FBS at standard cell culture conditions (37°C; 95% relative humidity and 5% CO2). Evaluation was done using the well-established MTT assay (24). Twenty four h after seeding the cells in 96 well-plates, media were removed and 0.01, 0.1 and 1 mg/mL of PLGA or PEG2PLA30 nanoparticles, redispersed in cell culture medium, were added. After 4 h of incubation at standard conditions, the cell culture medium was removed, and cells were washed once with PBS (pH=7.2). Afterwards, 200 μL of a MTT in PBS solution was added to each well (final concentration of 0.4 mg/mL), and cultures were again incubated for 4 h under the same conditions. Thereafter, the supernatant was carefully aspirated, and a solution of sodium dodecyl sulphate (10% in PBS) was added for cell lysis. For the dissolution of the precipitated formazan crystals, the well-plates were stored over 24 h at room temperature under exclusion of light. Subsequently, quantitative formazan concentration was determined by measuring the optical density at 550 nm with a background correction at 630 nm using an automatic microplate reader (TiterTek Plus, Germany). The water-soluble MTT is only converted into an insoluble formazan dye by the mitochondrial dehydrogenase by living cells (25). Cytotoxicity is expressed as cell viability of the treated cells relative to the untreated ones (negative control). A solution of Triton X 0.5% in PBS was used as a positive control; untreated cells with medium served as a negative control, and blank values were obtained from cell-free medium.

In Vivo Imaging and Analysis

Distribution, accumulation and elimination processes were studied by measuring the fluorescence signal of the respective dye which was incorporated into the nanoparticles. All procedures of the in vivo experiments complied with the standards for use of animal subjects as stated in the...
guideline from the animal care and use committee of Saxony Anhalt. All in vivo studies were performed in nude, female and male mice (Crl:SKH1-Hsd, 25–30 g) from Charles River Lab, which have humoral and cellular immunity comparable to C57Bl/6 mice (26). All mice were housed under controlled conditions (12 h light/dark schedule, 24°C).

An aliquot (2.0, 4.0, 6.0 and 6.5 mg polymer, 5% sucrose) of the freeze-dried fluorescent nanoparticles was redispersed in 1 ml of purified water and isotonised with sorbitol. Sixty μL of each sample were then slowly injected into the tail vein of non-narcotized mice. During the imaging procedure, a mixture of isofluorane/oxygen was used as anaesthesia gas with an initial flow of 4% isofluorane (3.0 L/min oxygen) and a steady-state flow of 1.8% isofluorane (1.5 L/min oxygen). All mice were placed under same conditions (stage height, mouse position, objective adjustment) on a 35°C temperature-controlled heating plate to prevent them for cooling out.

All in vivo fluorescence imaging measurements were carried out using the Maestro™ in vivo fluorescence imaging system (Cambridge Research & Instrumentation, Woburn, United States) and the Maestro™ software (version 2.10) (27). A Cermax®-type 300 Watt Xenon lamp with 5600 K, a NIR excitation filter (710 nm) and an 800 nm long-pass emission filter were used to detect DiR. The software acquired multispectral image cubes in 10 nm steps in the spectral range between 780 and 950 nm. The exposure times. Pixels with maximum intensities are set to 580 nm long-pass, acquisition setting: 550–800 nm) in 2 nm steps. Fluorescent imaging files were acquired during the first hour after injection. All other parameters accorded to the DiR measurements.

Ex Vivo Imaging and Analysis

For ex vivo analysis, the mice were sacrificed using carbon dioxide 24 h after injecting the nanoparticles. Excised organs were imaged with the Maestro™ in vivo imaging system. To ensure reproducible conditions like the arrangement of the organs, positioning in the Maestro™, incident angle of the light, etc., the organs or respective organ parts (intestine and liver in the size of the hole) were placed into a 24-holes well-plate. The imaging procedure accorded with the in vivo measurements. To detect potential minor accumulation in different organs, exposure times above the autoexposed values were used. Therefore, the liver was masked with a black plastic plate to inhibit an overexposure. For further calculation and evaluation, a region of interest (ROI) in the size of the holes of the well-plate was generated, and the corresponding exposure-time weighted total and maximum fluorescence signals were calculated.

To exclude the influence of different dye amounts due to varying nanoparticle concentration, the ex vivo total intensity value was divided through the maximum intensity of the in vitro emission spectra of the nanoparticles. These in vitro spectra were measured prior to injection, all in equal volumes and under the same measurement conditions. Afterwards, all values were normalized to 100% related to the highest fluorescence intensity.

RESULT AND DISCUSSION

Physicochemical Properties

Photon Correlation Spectroscopy (PCS)

PCS allows fast, robust and reproducible measurements of intensity weighed, hydrodynamic mean diameters of particles in the size range between about 5 nm and 2 μm. For the PEG-PLA nanoparticles, hydrodynamic diameters between 104 and 166 nm were measured (Table I and Fig. 1a). PDI values below 0.13 indicate that the nanoparticles are, compared to literature data, rather homogeneous in size (12). A higher polymer amount and the incorporation of DiR increased the particle size to about 60 nm and also slightly the PDI. PLGA nanoparticles were distinctly larger, and the high PDI (0.52) indicates inhomogeneity, which was already macroscopically visible. For the filtered (0.8 μm) dispersion, a z-average of 209 nm and a PDI of 0.15 were measured.
**Transmission Electron Microscopy (TEM)**

Visualization of two nanoparticle batches was carried out by TEM of negatively stained samples (Fig. 1c). The dimensions of the nanoparticles are shown in Table I. The sizes were generally smaller than those determined by PCS (30,31) and AF4/MALLS. However, PCS determines the hydrodynamic diameter of particles, which is sensitive to the hydrated PEG chains on the surface of the nanoparticles. In contrast, dried nanoparticles were viewed in TEM, and size calculations are weighted by the number of the particles. Bigger particles are less frequent in the prepared batch than smaller ones. Furthermore, in the light scattering methods larger sample volumes are measured compared to TEM. If there are bigger particles present in the batch, they have a distinct effect to the calculation in PCS, but they will probably not be detected with TEM.

**Asymmetrical Flow Field-Flow Fractionation (AF4)**

AF4 combined with MALLS allows accurate size evaluation due to sample separation prior size determination (23,32). The characteristic D10, D50 and D90 diameters of the mass-weighted size distributions are shown in Table I and Fig. 1a. PCS z-averages were larger than the median (D50) determined by AF4/MALLS. This is the result of the water binding between the PEG chains on the nanoparticle surfaces as discussed above. This influences the movement of the nanoparticles during the PCS measurements and thereby the detected nanoparticle size. Due to principle of MALLS measurements, which was applied to retrieve geometrical mass weighted particle sizes (RMS radius or radius of gyration) (33), the influence of the water shell is reduced. However, overall the D50 diameters confirm the size trend between the different nanoparticle batches measured in PCS and TEM. The larger difference between the PCS and MALLS results of batch D compared to batch B and C can probably be attributed to the higher sensitivity at both ends of the particle size distribution in the AF4/MALLS, which allows better quantification of smaller and larger particles in the sample. Size distribution was clearly broader in batch D compared to those of batch B and C (Fig. 1b). This is in good agreement with the higher PDI measured with PCS for batch D nanoparticles. The possibility of analyzing the amount of smaller and bigger particles in the dispersion is of great importance for meaningful interpretation of the in vivo results. The broad distribution of batch A (PLGA nanoparticles) with D90 values up to 400 nm is also visible in the cumulative size distribution (Fig. 1b), although the sample was filtered before the measurements. Due to this inhomogeneity in size as well as the absence of PEG, which would yield in higher accumulations in the RES (11,13), the PLGA nanoparticles were not studied in vivo.

**Stability Evaluation**

Potential physical instability and polymer degradation during storage in aqueous dispersion were investigated by PCS and AF4/MALLS. The results are shown in Table II. D10, D50 and D90 diameters (AF4/MALLS) are also given in Fig. 1a (marked with *). The size of both PEG-PLA nanoparticle batches stayed constant during storage. This is in accordance with literature data which has shown that such nanoparticles are comparatively stable for several months when stored at 5°C (34,35). They also reported in vitro studies at 37°C where they showed polymer and particle degradation within several months. This can be
Supplemental material
(b) Characterization of nanocarriers and their potential usage in cancer therapy

### Table II

<table>
<thead>
<tr>
<th>Batch</th>
<th>A&lt;sup&gt;20&lt;/sup&gt;</th>
<th>B</th>
<th>C &lt;sup&gt;PEG&lt;sub&gt;PLA&lt;/sub&gt;&lt;sub&gt;20&lt;/sub&gt;&lt;/sup&gt;</th>
<th>PEG&lt;sub&gt;PLA&lt;/sub&gt;&lt;sub&gt;40&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCS - Z-average in nm (PDl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>209 (±0.15)</td>
<td>113 (±0.09)</td>
<td>104 (±0.08)</td>
<td></td>
</tr>
<tr>
<td>3 Months</td>
<td>207 (±0.13)</td>
<td>113 (±0.09)</td>
<td>103 (±0.05)</td>
<td></td>
</tr>
<tr>
<td>MALLS D10 (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>127 ± 10.4</td>
<td>66 ± 0.9</td>
<td>62 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3 Months</td>
<td>121 ± 1.7</td>
<td>65 ± 1.2</td>
<td>62 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>MALLS D50 (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>222 ± 9.0</td>
<td>95 ± 0.2</td>
<td>86 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>3 Months</td>
<td>208 ± 1.6</td>
<td>95 ± 0.4</td>
<td>86 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>MALLS D90 (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>403 ± 29.8</td>
<td>143 ± 0.6</td>
<td>132 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>3 Months</td>
<td>377 ± 1.7</td>
<td>140 ± 0.7</td>
<td>132 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a) The numbers 2 and 20 refer to the molar mass of the polymer block (kDa)</sup>

<sup>b) Sample was filtered (pore size 0.8 µm) prior measurements</sup>

assigned to possible in vivo behaviour and attest comparatively slow particle degradation. The high reproducibility of both PCS and AF4/MALLS results underlines the narrow distribution of the produced nanoparticle batches. The AF4/MALLS size results of (filtered) PLGA nanoparticles showed a slight decrease of the particle size, mainly in the upper size range, which may be an indication of starting polymer degradation (marked with black arrow in Fig. 1a). It has been reported that the rate of PLGA degradation is slightly increased in bigger particles, although this was more pronounced at higher temperatures (35). Overall, results indicate accurate stability of the nanoparticles in the dry state and even in aqueous dispersion, which makes them ideal for further applications.

**In Vitro Cytotoxicity Assay**

More than 90% of the cells were still viable after treatment with the nanoparticles in a concentration range between 0.01 and 0.1 mg/mL. This is in good accordance with literature data (36). Batch A and B (PLGA and PLA<sub>20</sub>, PEG<sub>20</sub>) nanoparticles might thus be recognized as biocompatible, which was the requirement for further in vitro experiments. Good biocompatibility can also be assumed for the PLA<sub>20</sub>PEG<sub>40</sub> nanoparticles due to the similar chemical composition.

**In Vivo Imaging**

Dialkylcarbocyanine dyes like DiI and DiR are widely used as tracers in living and fixed tissues, cells and in vivo imaging experiments (37–39). The emission spectrum of the dyes is very broad, which facilitates exact detection also in vivo. Furthermore, they are very stable, have low bleaching properties (38) and remain fluorescent also in vivo for up to one year (40). Dyes with fluorescence emission in the NIR region (700–900 nm) are required for detailed in vivo characterizations, particularly in deeper tissues (18). Biological tissues have a high photon absorbance of fluorescence emission light in the visible wavelength range (350–700 nm), mainly caused by hemoglobin but also in the upper infrared range (above 900 nm) due to the presence of water (41). In contrast, many tissues are optically transparent in the narrow spectral NIR area. DiR is a favorable dye with a fluorescent emission in the near infrared. Furthermore, as DiR is very lipophilic (42), it is tightly incorporated between the lipophilic PLA chains into the nanoparticles. Representative, measured in vitro and extracted in vivo emission spectra of DiR and of NR are shown in Fig. 2a. Compared with literature data, the in vivo emission maxima of DiR is slightly shifted bathochrom to higher wavelengths. This is caused by the cut-on of the emission filter set of the Maestro™ imaging system. Thus, for DiR the emission maximum was measured in vitro at about 818 nm instead of the manufacturer’s and published information of about 775 nm (38). However, this effect has no influence on the results and data interpretation, since all spectral analyses were only done with the Maestro™ system and the respective in vitro and in vivo reference spectra.

It is furthermore visible that the DiR emission spectra were similar in vitro and in vivo, indicating that the penetration of emitted fluorescence light through living tissue has no influence on the spectra profile, thus allowing exact detection of nanoparticles in vivo. In contrast, a spectral shift is visible between the in vitro and in vivo spectra when NR was incorporated into the nanoparticles (batch E, Fig. 2a). This finding can be attributed to the influence of the local polarity on the NR emission maxima. Increasing polarities result in a shift of the emission maxima to higher wavelengths and a decrease of the quantum yield. NR is nearly non-fluorescent in pure aqueous media (43).

**Figures 2b and c** show the images of a mouse 15 and 40 min after injection of nanoparticles loaded with NR (batch E), indicating that in vivo imaging of the nanoparticle distribution is in principal possible also with non-NIR dyes. However, nanoparticle accumulation in liver and spleen could not be detected due to the above-mentioned high...
Supplemental material

(b) Characterization of nanocarriers and their potential usage in cancer therapy

2002 Schädlich et al.

Fig. 2 (a) Normalized fluorescence in vitro and extracted in vivo emission spectra of NR and DiR. The DiR spectra are virtually overlapped. (b-e) Fluorescence intensity images of two mice 15 (b, d) and 40 min (c, e) after injecting NR (b, c) or rather DiR nanoparticles (d, e).

The very lipophilic properties of the DiR dye prevents a release from the nanoparticles. This is confirmed by other groups who showed that dialkylcarbocyanine is not or is only very slowly released from lipophilic nanocarriers (44, 51). Overall, these results confirm the expectation that for accumulation studies highly lipophilic dyes have to be incorporated to image the fate of nanoparticles in detail. The results after injecting DiR nanoparticles are shown in Fig. 2d and e. An accumulation in the bladder was not detected, as expected.

Distribution Studies

Tests about the in vivo fate of nanoparticles as well as the constraints of detection the distribution and accumulation were started by intravenous (i.v.) injection of batch D nanoparticle dispersions. The animals were imaged at various time points (i.e., 5 min after injection, 2 days). The visualized information from the abdomen of a mouse is shown as an inverted grayscale image in Fig. 3. Black is allocated to high DiR emission intensities and white to the background signal. Five min after injection, the blood vessels are clearly visible (Fig. 3b). During the first 6 h these vessels were still detectable, but sharpness and fluorescence...
intensity decreased slowly. This is in agreement with literature data where the plasma half-life time of about 6 h is given (32). Furthermore, there are two black parts in the image (marked by white arrows). They point to a part of the tail as well as the spleen at the right side of the mouse. A nanoparticle accumulation in the tail can be assigned to the injection procedure, where the tail is slightly fixed by hand to immobilize it. This may result in micro lesions where nanoparticles can pass the vascular wall resulting in a nanoparticle accumulation near the injection site. After 48 h (Fig. 3c), no defined fluorescent areas are identifiable anymore. All nanoparticles are eliminated or at least accumulated in RES body structures.

Due to the resolution of the CCD camera chip, analysis of the blood vessels on their own to calculate blood half-life times was not possible. Instead, the total signal of the whole body from five mice was measured at different time points (Fig. 3a). The calculated values are the sum of all subcutaneous dye signals (from blood vessels, upper parts of liver and spleen). They indicate that 50% of the initially detectable nanoparticles were eliminated after about 45 min and 75% after 95 min. However, as the blood vessels were detectable during the first 6 h, sufficient nanoparticles were still circulating. It is widely known that PLA nanoparticles without surface modification or ones which are only stabilized with poloxamer 188 have a half-life of just a few minutes (52,53). Due to the fact that PEG chains can adopt brush-like structures, they have the ability to reduce phagocytosis in vivo (54). The prolonged circulation due to reduced phagocytosis and opsonisation yields a subsequently reduced clearance by the reticuloendothelial system (RES). Consequently, nanoparticles are not eliminated as fast and may accumulate in, e.g., tumor tissues (1,7).

**Accumulation Studies**

Nanoparticle distribution and accumulation was followed in more detail by imaging the abdominal site of female mice at different time points (Fig. 4a-h). Five min after injection the whole body of the abdomen is homogeneous colored blue with some brighter blue parts in the chest (Fig. 4a), confirming that nanoparticles circulate homogeneously through the body and accumulate in the RES as a part of the immune system where the phagocytic cells are located in reticular connective tissues. After 4 h, the maximal fluorescence is visible in the area of the liver (Fig. 4b). Accumulation of the DiR nanoparticles in the spleen is also visible in the right part of the image (marked by arrow). Accumulation in the spleen could clearly be shown in the jet color images where the color is scaled to the respective intensity (Fig. 4c and d). Figure 4e shows the homogeneous fluorescent liver and the minor fluorescent spleen (marked by an arrow). During the next few days, nanoparticle accumulation in the RES decreased continuously, but fluorescence was still detectable after five days (Fig. 4d). *In vitro* tests have shown that the polymer degradation strongly depends on temperature and accelerates when temperature is increased, e.g., from 5 to >25°C rapidly (34, 35). However, even at 37°C, complete polymer degradation takes still more than one month depending on the dissolution media. Therefore, it can be assumed that the nanoparticles will be also slowly disintegrated *in vivo* and can be detected in the jet color images even five days after injection (Fig. 4h).

**Ex Vivo Imaging**

*Ex vivo* studies could provide more detailed information about the nanoparticle accumulation in the different tissues. Mice were sacrificed 24 h after nanoparticle injection, and the respective organs were placed in a 24-holes well-plate (Fig. 4i). Measuring the viscera of an untreated mouse resulted in a black image without any detectable fluorescence signal as shown in Fig. 4j. This control confirms the selectivity in detecting the DiR dye using fluorescence imaging. Figure 4k shows the isolated organs. As expected, high fluorescence intensity was visible in the liver but could also be detected in small parts of the ovaries. No signals were visible in the other organs. However, after masking the liver tissue with a black plastic plate, which allowed higher exposure times, nanoparticle accumulation could also be visualized in the intestine, uterus and spleen (Fig. 4l). The absence of fluorescence in the kidneys indicates that the nanoparticles as well as the dye are not eliminated by urine. This was expected due to the highly lipophilic character of the dye and the size of the nanoparticles. Very lipophilic molecules are often eliminated by bio-conjugation in the liver and excretion via the gall into the intestine (55). Afterwards, they are excreted with the feces. This elimination route was confirmed by the detected fluorescence in the intestine in Fig. 4l.

By implementing a round region of interest (ROI) in the size of the well-plate hole (cp. Fig. 4k) measuring different parameters of each organ became possible, determining total, maximum and average signal intensities, which are related or not related to the respective exposure times. By detailed analysis of this data, quantitative information can be obtained. Maximum and total signal intensities correlated well to the exposure times and were most suitable for further calculations. The maximum intensity values allow the comparison of nanoparticle accumulation in organs differing in size. For example, a high enrichment in a part of a tissue leads to high maximum intensity values within the ROI. The total signal of the same sample, as the sum of all pixels in the ROI, would be, however, only slightly...
increased. Nevertheless, the total signal is a value with lower variability between different measurements. The normalized maximum and total intensities from selected organs are shown in Fig. 5. Neither exposure time nor usage of the black plastic plate had an influence on the results (except the liver). Thus, potential errors caused by different exposure times which are not in the optimum range of the respective organ can be excluded, and results obtained by automatically set exposure times can be compared. It is also worth noting that the standard deviation is much larger for the maximum signal (Fig. 5b) compared to total intensity (Fig. 5a).

As shown in Fig. 5a, ex-vivo results confirm the nanoparticle accumulation in the RES organs liver and spleen as already detected in vivo. No total intensity was noticed in fat tissues and the lungs, and only slight signals were present in the intestines and in the kidneys. Overall, the same trend was found when analysing the normalized maximum intensities (Fig. 5b). Interestingly, a maximum signal of about 25% was also found in the lung. However, a distinct accumulation of the nanoparticles in the lungs appears not to be due to the standard deviation. This was very high and there was neither a signal found in the evaluation of the total signal nor in the images as shown in Fig. 4l.
How Stealthy are PEG-PLA Nanoparticles? An NIR In Vivo Study

CONCLUSION

This work demonstrates the preparation, characterization and application of PEG-PLA block polymer nanoparticles with defined sizes and narrow size distribution. To prolong the in vivo circulation time, PEG with an average molecular weight of 2000 was covalently bound to PLA,

viscera like fat tissue and kidneys showed maximum intensity levels of 10% or less, which is in the same range of the masked liver values. This can be attributed to the detection limits using the maximum intensity values. Intensities of about 15% were found in the intestine, confirming the image results (Fig. 4) as well as the calculated total intensity (Fig. 5a). The different fluorescence ratios (liver to spleen) of about 5:1 for the total signal and 2:1 for the maximum are due to differences in the size of the organs. The spleen is much smaller than the liver.

The influence of the nanoparticle concentration on their biodistribution is shown in Fig. 6. These experiments were carried out to evaluate the influence of varying nanoparticle amounts on the accumulation behaviour and to determine the detection limits for imaging. The injection of 60 μL with a nanoparticle concentration of 2.0 mg/mL (injected mass 0.12 mg) did not allow meaningful data analysis due to the too low fluorescence intensity. The range between 4.0 and 6.5 mg/mL (injected mass between 0.24 and 0.39 mg) yielded reproducible results with an average variation of about 15% or less. The total signals were independent of the sex of the mice. However, the age of the mice may be an important factor: Total signals in the spleen were higher in the mice that were three times older (Fig. 6), probably due to the increase in organ size with age. Age dependence was not observed in the liver signal, but this organ is bigger than the hole of the well plate, and only a part of the liver (similar in size for all mice) was studied.
and nanoparticles composed of PEG-PLA block copolymers could be prepared. Nanoparticles were lyophilized to assure long-term stability, but even the aqueous dispersions were stable over at least three months when stored at 5°C. PEG-PLA nanoparticles were biocompatible and non-toxic to CHO and L929 cells, and detailed particle size characterization by PCS, AF4/MALLS and TEM provided accurate size information. AF4/MALLS results showed that even in batches with rather low polydispersity indices (PCS z-average = 166 nm, PDI = 0.13, batch D), 10% of the nanoparticles were smaller than 48 nm (D10) and the same amount larger than 230 nm (D90). This confirms the need to combine different size measurement techniques for detailed particle size characterization as consumption for size-dependent biodistribution studies and underlines the necessity of preparing and characterizing homogeneous nanoparticles for further in vivo experiments.

First, in vivo experiments with NR-loaded nanoparticles indicated a fast release of the dye from the particles after injection, resulting in rapid dye elimination from the bloodstream. In contrast, incorporation of the highly hydrophobic DiR allowed direct detection of the nanoparticles in the blood stream for up to 6 h. Consequently, these nanoparticles will have sufficient time to accumulate in tumor tissue due to the EPR effect. Detailed information about nanoparticle accumulation in various organs was obtained by fluorescence imaging and measuring the total and maximum fluorescence signals in the organs ex vivo. Based on our results, the combination of these values and the comparison with the in vivo imaging data appears to be a promising approach to study nanoparticle accumulation in different organs. Furthermore a new calculation approach was described, which allows the comparison of nanoparticle batches with varying fluorescence intensities. This provides the basis for the determination of the influence of varying particle sizes on the in vivo fate. In the future, tumor accumulation should be studied using PEG-PLA nanoparticle batches with different sizes but narrow and well-established size distribution in two tumor models with different shapes and growth rates: the colon carcinoma (HT29) and the ovarian carcinoma (A2780).

ACKNOWLEDGMENTS

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Tumor Accumulation of NIR Fluorescent PEG—PLA Nanoparticles: Impact of Particle Size and Human Xenograft Tumor Model

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Unfortunately, a high percentage of potent drug candidates for anticaner therapy are poorly soluble in water.¹² Many of them possess a polycyclic structure which enhances the ability of the molecules to reach cellular targets.² Owing to their hydrophobic characteristics many of these therapeutic agents never enter the formulation development stage.¹ This remains one of the main challenges in future anticancer chemotherapy. Nanoparticles with a hydrophobic matrix provide an excellent possibility for the formulation of such poorly water-soluble drugs. The biodegradable and biocompatible polyesters poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLGA) are frequently used for this purpose and are approved in several market products for parenteral application by the FDA.⁴ PLA is more hydrophobic as compared to PLGA. Therefore, PLA nanoparticles can homogeneously incorporate very lipophilic drugs in their core. After intravenous (i.v.) application and possible accumulation into tumor tissue the slow degradation of PLA⁵,⁶ provides a continuous release of the drug at the place of its action. Such nanoparticles thus present an interesting approach for a convenient therapy with extended drug administrations. However, the nanoparticles have to reach and accumulate in tumor tissues where the drug is needed. Both steps, transport via the bloodstream as well as tumor enrichment are triggered by two main parameters, the particle size and the surface properties of the nanoparticles.

It is well established that flexible hydrophilic surfaces like polyethylene glycol (PEG) have the ability to extend circulation time and retention half-life of nanoparticles as they reduce opsonisation and the subsequent clearance by the mononuclear phagocytic system (MPS) also known as reticuloendothelial system (RES). The RES has been shown to be the major barrier for an effective tumor targeting using nanoparticles.⁷ Thereby, mainly the Kupffer cells of the liver play a key role in the elimination of hydrophilic nanoparticles.

**ABSTRACT**

Cancer therapies are often terminated due to serious side effects of the drugs. The cause is the nonspecific distribution of chemotherapeutic agents to both cancerous and normal cells. Therefore, drug carriers which deliver their toxic cargo specific to cancer cells are needed. Size is one key parameter for the nanoparticle accumulation in tumor tissues. In the present study the influence of the size of biodegradable nanoparticles was investigated in detail, combining in vivo and ex vivo analysis with comprehensive particle size characterizations. Polyethylene glycol—polymers poly-(lactide) block polymers were synthesized and used for the production of three defined, stable, and nontoxic near-infrared (NIR) dye-loaded nanoparticle batches. Size analysis based on asymmetrical flow field fractionation coupled with multangle laser light scattering and photon correlation spectroscopy (PCS) revealed narrow size distribution and permitted accurate size evaluations. Furthermore, this study demonstrates the constraints of particle size data only obtained by PCS. By the multispectral analysis of the Maestro in vivo imaging system the in vivo fate of the nanoparticles next to their accumulation in special red fluorescent DsRed2 expressing HT29 xenografts could be followed. This simultaneous imaging in addition to confocal microscopy studies revealed information about the accumulation characteristics of nanoparticles inside the tumor tissues. This knowledge was further combined with extended size-dependent fluorescence imaging studies at two different xenograft tumor types, the HT29 (colorectal carcinoma) and the A2780 (ovarian carcinoma) cell lines. The combination of two different size measurement methods allowed the characterization of the dependence of nanoparticle accumulation in the tumor on even rather small differences in the nanoparticle size. While two nanoparticle batches (111 and 141 nm in diameter) accumulated efficiently in the human xenograft tumor tissue, the slightly bigger nanoparticles (diameter 166 nm) were rapidly eliminated by the liver.

**KEYWORDS:** In vivo imaging · fluorescence imaging · AF4 · PEG—PLA · nanoparticle · HT29 · A2780
as well as the macrophages of the spleen have been identified to be responsible for the rapid clearance of nanocarriers from the bloodstream.7,8 Tumor tissue is often characterized by a leaky vasculature with an enhanced permeability.7,9 Intravenously injected nanoparticles can accumulate by passive diffusion due to this hyper-permeable tumor vasculature. This retention of nanoparticles in the tumor tissue is called EPR (enhanced permeability and retention) effect.10 Individual molecules like drugs and dyes are transported through the bloodstream for only a few minutes up to 1 h until they are eliminated rapidly. In contrast, properly designed, nanoparticles can reach much longer circulation times and have the ability to be retained in tumor tissues due to the EPR effect. It has been shown that the upper size limit for extravasation into solid tumors is about 400 nm.11 Other groups showed that particles with diameters <200 nm accumulate even more effectively than bigger ones.9,12,13 This can be explained by an increased nanoparticle uptake by the RES in the size range between 150 and 300 nm.12 A lower size limit based on size is difficult to define due to further influencing parameters like structure, surface charge, and molecular flexibility.15 Studies have shown that the size limit for renal excretion of proteins and water-soluble polymers is approximately 45 kDa (hydrodynamic diameters <8 nm), depending on the particle size, shape, density, and the surface charge.15–18 It is furthermore known, that also liposomes which are smaller than 70 nm are faster cleared from the bloodstream than larger ones.19,20 This is the effect of extravasation and accumulation in the parenchymal cells of the liver.20 On the basis of this knowledge and of other reports, the optimum nanoparticle size for tumor accumulation is between about 70 and 200 nm.21 This rather narrow size range clarifies the necessity of the preparation of nanoparticle formulations with well-defined and characterized sizes and sufficiently narrow size distributions. Only some few publications discuss nanoparticle-dependent tumor accumulation and in vivo biodistribution, however, based on particle batches with polydispersity indexes (PDI) between 0.2 and 0.5.22,23 Because of the broad particle size distribution in these studies, information about the in vivo fate can only be drawn with care. This again underlines the necessity to control the size of the nanoparticles during preparation. In addition, reliable and appropriate size determinations using different size measurement techniques are necessary as the basis for meaningful interpretation of in vivo data.24

The aim of the present work was to investigate the in vivo fate and tumor accumulation of three PEG2-PLA20 or PEG5-PLA20 (numbers in kDa) nanoparticle formulations with different and defined sizes. Storage stability, homogeneity after redispersing, and sufficient stability in aqueous media as well as high biocompatibility were already established, and the results evidenced the high potential of these nanoparticles as drug delivery systems.25 In addition, nanoparticle sizes were studied in detail using asymmetrical field flow field fractionation (AF4) coupled with multi-angle laser light scattering (MALLS) and photon correlation spectroscopy (PCS). These size evaluations were the main requirement to correlate the influence of particle size on the in vivo distribution, studied by near-infrared (NIR) fluorescence imaging.25 This imaging technique provided a noninvasive monitoring modality with high temporal resolution also in deep tissues.26 Fluorescence imaging, especially the Maestro in vivo imaging system allowed the detection of multispectral emission curves and also the exclusion of the autofluorescence from the mice.27,28 Hence, contemporaneous imaging analysis of fluorescent tumors, dyed nanoparticles and autofluorescence of the mice was possible.

In the present study, tumor accumulation of PEG–PLA nanoparticles differing in size was evaluated on two different xenograft tumor types simultaneously to improve the understanding of the in vivo nanoparticle tumor targeting capacity. Xenograft tumors derived from the HT29 colon carcinoma cell line and the A2780 ovarian carcinoma cell line were selected due to their differences in structural shape and growth.

To the best of our knowledge, it is the first time that the influence of size of narrow distributed nanoparticle batches were studied in detail using AF4/MALLS as well as PCS and combined with extensive biodistribution studies. The results of this study provide information about the influence of the particle size on the in vivo fate and tumor accumulation behavior of PEG–PLA nanoparticles up to the cellular level. This study serves as the basis for further nanoparticle applications to enhance the therapeutic activity and safety of chemotherapeutic agents.

RESULTS AND DISCUSSION

Nanoparticle Preparation. By variation of the polymer concentration in a fixed volume of the organic solvent, the particle size of the nanoparticles can be controlled. Higher polymer concentrations lead to increased particle sizes. On the other hand, the ratio of the external aqueous phase or the amount of the polymer solution (at fixed polymer concentration) had nearly no effect on the size of the produced nanoparticles.29 On the basis of this knowledge, different amounts of PEG–PLA were used to produce three batches of DiR-loaded nanoparticles with different sizes (Table 1). One unloaded batch was produced for extended size characterization (batch D). Previous studies on the physical stability and polymer degradation attested the nanoparticles to be stable in the dried state for more than 6 months. The nanoparticles were even
stable in aqueous dispersion for up to 3 months when stored in the refrigerator at about 5 °C, and no agglomeration or degradation was observed.25 These findings are in good accordance with literature data.29,30

**Physicochemical Properties.** Fluorescence Spectroscopy. Fluorescence intensities of the different nanoparticle batches are shown in Table 1. Lowest intensities were measured for batch A nanoparticles due to the lower DiR-load of these nanoparticles. Batch B showed highest fluorescence intensities indicating high incorporated dye amounts. The intensity of batch C was in between where the dye loading was decreased due to the higher amount of polymer.

**Photon Correlation Spectroscopy (PCS).** The hydrodynamic mean particle diameters (z-averages) are presented in Table 1. The results confirmed the production of nanoparticles with different sizes. The size range was between 111 nm (batch A) and 166 nm (batch C), hence within the size range which is reported to be ideal for cancer treatment (70–200 nm).21 All PDI values were between 0.13 and 0.16 indicating an overall narrow particle size distribution.

**Asymmetrical Flow Field Flow Fractionation (AF4).** AF4/MALLS measurements allowed an accurate determination of size distributions due to the fractionation step prior to size determination.31,32 The calculated D10, D50, and D90 diameters of all three batches are shown in Table 1. The D50-diameters (median) were considerably smaller compared to the PCS z-averages. This was caused by geometrical mass weighed particle sizing in AF4 measurement which reduced the influence of the water shell and the presence of minor amounts of larger particles in all batches. All three nanoparticle batches had small fractions of larger particles (>200 nm) but no particles with diameters larger than 600 nm could be detected.

Nevertheless, the LS detector signals over the whole elution time as well as the corresponding cumulative mass distributions (Figure 1) confirmed the overall narrow size distribution of the nanoparticle batches. The analysis of batch A indicated that the predominant fraction of nanoparticles was in the size range between 36 and 80 nm. Only less than 10% were larger than 100 nm (Table 1). Size distribution was broadest for batch C. The amount of nanoparticles which was smaller than 48 nm was similar to that with sizes larger than 230 nm. The results indicated that PCS z-averages are mainly influenced by larger particles. This underlined the advantages of AF4 to get more detailed information of the nanoparticle size distribution which has a major influence to possible in vivo behavior.

**Nanoparticle Stability in FBS and PBS Containing Media.** The PCS and AF4 measurements were extended to investigate the influence of physiological conditions to the particle size distribution (Table 2). The z-average in water was determined to be 112 nm with a PDI of 0.08 indicating an even narrower distribution than that of the loaded nanoparticles. Consequently, the D50 value of 100 nm based on the AF4 results was comparable to the PCS result. The difference of 12 nm can be attributed to the water binding between the PEG chains, which influence the movement of the particles in the PCS measurement. The addition of PBS had no influence on the z-average although the

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**Table 1. Nanoparticle Compositions and Particle Sizes of Freshly Dissolved Nanoparticle Batches**

<table>
<thead>
<tr>
<th>Batch</th>
<th>Polymer Composition</th>
<th>A. PEG/PLA</th>
<th>B. PEG/PLA</th>
<th>C. PEG/PLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIR loading</td>
<td>0.5%</td>
<td>1%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Polymer concentration</td>
<td>0.75%</td>
<td>1%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>PCS, z-average in nm (PDI)</td>
<td>111 ± 2 (0.10)</td>
<td>141 ± 1 (0.13)</td>
<td>166 ± 2 (0.13)</td>
<td></td>
</tr>
<tr>
<td>MALLS D10 (nm)</td>
<td>36 ± 2</td>
<td>40 ± 1</td>
<td>48 ± 10</td>
<td></td>
</tr>
<tr>
<td>MALLS D50 (nm)</td>
<td>43 ± 2</td>
<td>64 ± 1</td>
<td>99 ± 4</td>
<td></td>
</tr>
<tr>
<td>MALLS D90 (nm)</td>
<td>80 ± 3</td>
<td>153 ± 1</td>
<td>230 ± 2</td>
<td></td>
</tr>
<tr>
<td>Fluorescence intensity</td>
<td>~32</td>
<td>~100</td>
<td>~76</td>
<td></td>
</tr>
</tbody>
</table>

| Note: | The number 2, 20, and 40 refers to the molar mass of the polymer block (kDa). |
| Note: | Dye loading in percent related to the polymer amount. |
| Note: | Polymer concentration in percent related to the organic solvent. |
| Note: | Data is based on previously shown results.25 |
**Table 2. Particle Size of PEG2Pla Nanoparticles in Different Dispersing Media**

<table>
<thead>
<tr>
<th>Dispersing Media</th>
<th>D10 (nm)</th>
<th>D50 (nm)</th>
<th>D90 (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>112.0 (0.08)</td>
<td>69</td>
<td>100</td>
</tr>
<tr>
<td>Water (10% FBS)</td>
<td>112.3 (0.10)</td>
<td>71</td>
<td>101</td>
</tr>
<tr>
<td>Water (10% FBS)</td>
<td>91.1 (0.29)</td>
<td>71</td>
<td>101</td>
</tr>
<tr>
<td>Water (10% FBS, 10% PBS)*</td>
<td>90.8 (0.30)</td>
<td>71</td>
<td>101</td>
</tr>
</tbody>
</table>

*Particle sizes were determined 24 h after incubation.

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**Figure 2.** (A) Grayscale intensity confocal microscopic images of HT29 tumor cells (incubated with batch A and untreated). (B) Carcinoma cells, incubated with batch A nanoparticles (red) and stained by membrane dye DiO (untreated). (C) HT29 tumor model. (2011), 8710-8720.

**Figure 3.** Visualization of nanoparticle accumulation in tumor models. Nanoparticle accumulation was studied in male nude mice bearing two subcutaneous (s.c.) HT29 xenograft tumors, one on each flank. For clear visualization of nanoparticle accumulation in the tumor tissue and normal nanoparticle distribution within the body, DiSRed2 expressing HT29 cells were used. This allowed multispectral in vivo imaging of dye NIR nanoparticles (red) and the fluorescent tumor cells (green) simultaneously (Figure 3A). At 10 min after i.v. application of NIR fluorescent nanoparticles high fluorescence intensities were detected in the s.c. blood vessels in the abdominal area. Also the blood vessels above both tumor grafts (green fluorescence) were well visible. The intensity of the circulating particles decreased continuously with time after injection and were detectable in the blood vessels for about 6 h. Afterward the intensity fell below the detection limit of the fluorescence imager.25

The scaled intensity images (Figure 3B) were obtained by using the “compare imaging” function of the extracted DiR signal. This allowed time dependent visualization of nanoparticle accumulation in tumor tissues. Different measurement conditions like varying exposure times were recomputed by the software and all four images were displayed in relation to each other.

The big blood vessels under the skin were well visible within the first minutes after injection (Figure 3B). Already 3 h later there was a clear accumulation in the area of the tumor accompanied by a simultaneously decreased intensity in other parts of the body. The enrichment within the HT29 tumors increased distinctly up to 24 h (Figure 3B). This was probably caused by the EPR effect by what the passive accumulation of nanocarriers occurs after a single application during the first 24 h.35,36 After 48 h the DiR intensity in the tumor decreased continuously but remained visible with clear differences in the fluorescence intensities. The results indicate that the nanoparticles have the ability to bind to or to accumulate in the cells. On the basis of these experiments, the tests were repeated using both A2780 cells, the second cell line used in vivo, and HT29 cells. Cell membranes were stained with DiO for better localization of fluorescent nanoparticles. Overall, the results indicate that the nanoparticles were mainly bound to the cell membranes in both cell lines but with a higher association tendency to A2780 tumor cells (Figure 2B). This might be caused by the PEG shell which inhibits the nanoparticle uptake into the cells.34 But it has to be kept in mind that PEG is necessary to circumvent nanoparticle recognition by the RES. If they are fast eliminated by the liver they will not reach tumor tissues and in vitro observed nanoparticle uptake will fail in vivo. But even nanoparticles, which stick on the surface, will slowly be eroded and degraded. Thereby, the dyes or drugs will be released and diffuse into the cells effectively.

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**In Vivo Accumulation Studies in DiSRed2 Fluorescent HT29 Tumors.** Nanoparticle accumulation was studied in male nude mice bearing two subcutaneous (s.c.) HT29 xenograft tumors, one on each flank. For clear visualization of nanoparticle accumulation in the tumor tissue and normal nanoparticle distribution within the body, DiSRed2 expressing HT29 cells were used. This allowed multispectral in vivo imaging of dye NIR nanoparticles (red) and the fluorescent tumor cells (green) simultaneously (Figure 3A). At 10 min after i.v. application of NIR fluorescent nanoparticles high fluorescence intensities were detected in the s.c. blood vessels in the abdominal area. Also the blood vessels above both tumor grafts (green fluorescence) were well visible. The intensity of the circulating particles decreased continuously with time after injection and were detectable in the blood vessels for about 6 h. Afterward the intensity fell below the detection limit of the fluorescence imager.25

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over several days as shown exemplarily in Figure 3B. This result is in agreement with the understanding of the EPR effect.10 Owing to polymer degradation at 37 °C which is reported to take in vitro more than 1 month19,30 we expect a continued long-term release of the dye from the nanoparticles.

**Ex Vivo Accumulation Studies in DsRed2 Fluorescent HT29 Tumors.** A DsRed2 fluorescent tumor bearing mouse was sacrificed 48 h after nanoparticle injection (Figure 3B). The tumor was imaged ex vivo using the Maestro (Figure 3C). Analyzing the cube to assign DiR and DsRed2 signals allowed the generation of respective corresponding jet color intensity images. The DsRed2 image (Figure 3C) revealed clusters with high fluorescence intensity of DsRed2 expressing cell clusters. DiR jet color image in Figure 3C illustrated the fluorescent areas where DiR nanoparticles accumulated. Highest concentrations were located between DsRed2 expressing cells. This was confirmed by the overlay image (Figure 3C). The highest threshold values of the DiR signal were displayed red, and this information was afterward overlaid on the green displayed DsRed2 areas.

After ex vivo imaging a sliced tumor part was analyzed by confocal microscopy. By this, the detection of NIR fluorescent nanoparticles in addition to the DsRed2 expressing tumor cells was possible. Figure 3D shows the plain-imaged tumor suspension for comparison. The red-appearing DiR-loaded nanoparticles were homogeneously distributed and scaled in the nanometer size range. Compared to the size results obtained by PCS and AF4/MALLS, the particles appeared bigger due to also laterally emitted fluorescence light. Examination of the sliced tumor tissue (Figure 3D) allowed visualization of the PEG–PLA nanoparticles (red spots) between the xenograft tumor cells (green). Most nanoparticles were located in non-DsRed2 fluorescent, channel-like areas. These consist either of necrotic tumor tissue or of nonfluorescent mouse cells. On the basis of additional performed hematoxylin and eosin (H&E) analyses, an accumulation of nanoparticles mainly in necrotic/fibrotic tumor tissue was proven.

**In Vivo Fluorescence Imaging Studies.** Nonfluorescent HT29 and A2780 cell lines were used as xenograft models to evaluate tumor accumulation dependent on the nanoparticle size. The HT29 tumors are characterized by their bright and firm shape; they grow slower than A2780 tumors and generally contain large central necrotic/fibrotic areas which are surrounded by peripherally arranged vital tumor cells.37 The A2780 tumors grow faster and are highly vascularized. Their
blue color attests a high perfusion and vascular permeability which is more than 4 times higher than that in neighboring tissues. Owing to the smaller amount of connective tissue, A2780 tumors are more soft compared to HT29 tumors.

For direct comparison, both of these xenograft tumors were established in the same mouse, one to the right and to the other to the left side. The A2780, known as a fast growing tumor, reached in all groups average tumor sizes between 1.25 and 2.25 cm\(^3\). They were 3–6 fold bigger than the HT29 ones (0.3–0.5 cm\(^3\)) and showed larger size variations within the respective group. All mice were imaged for 24 h after nanoparticle injection of batches A, B, and C (Table 1). The resulting \textit{in vivo} images yielded overall similar nanoparticle distributions as shown in Figure 3A,B. NIR nanoparticles accumulated slowly but continuously in the tumor during the first 24 h after injection. Nanoparticle tumor accumulation was clearly detectable \textit{in vivo} in more than 80% of the mice. However, pharmacokinetic data and tumor enrichment differences between all three nanoparticle batches could not be visualized due to the limited resolution of the fluorescence images.

\textit{Ex Vivo Fluorescence Imaging Studies.} The distribution pattern inside both tumor models was further investigated by the comparison of fluorescence images of complete and sliced tumor tissues. The results of group A are exemplarily shown in Figure 4D,E. Nanoparticles were highly enriched in all three A2780 tumors (Figure 4 D). In contrast the smaller HT29 tumors showed no or only slight fluorescence, whereas the bigger one was highly fluorescent (Figure 4 E). After cutting the HT29 tumor into two pieces, inhomogeneous fluorescence of accumulated nanoparticles was visible. Two areas with highest fluorescence intensity could be identified: the boundary and the central area. On the basis of Figure 4D an irregular distributed nanoparticle accumulation in the A2780 tumor tissue can be assumed. Different inner and surface parts are dark-red. In contrast there are large areas appearing dark-blue with only low fluorescence intensity, indicating areas with less accumulated nanoparticles. Dark-red and dark-blue areas as found in the A2780 tissue (Figure 4D) indicating only very high or minor nanoparticle concentrations could not be seen in the HT29 tumors (Figure 4E). This confirmed a more homogeneous nanoparticle distribution throughout the tumor. The low interstitial pressure in parts of the HT29 tumor tissue causes that after extravasation, the nanoparticles diffuse and accumulate in the tumor center (Figure 4E). As it is visible in the H&E stained light microscopic images shown in Figure 4G, this is an area with low central microvessel densities but with central fibrotic and or necrotic areas which is in accordance with literature.\textsuperscript{37,39,40} Jain et al. characterized the

![Figure 4. Normalized total fluorescence intensity amounts (A, B) and maximum intensities (C). (A) Time-dependent nanoparticle accumulation in A2780 tumors, \textit{in vivo} measured compared to 24 \textit{ex vivo} results. (B and C) Tumor size related fluorescence intensities of HT29 and A2780 tumor accumulation values, 24 h after i.v. injection, measured \textit{ex vivo} (SD, n = 3). (D and E) \textit{Ex vivo} fluorescence images of excised (left) and sliced (right) xenograft tumors (group A, 24 h after i.v. injection). (F and G) Light microscopic images of excised and sliced xenograft tumor tissues (H&E stained). Arrows point to blood vessels (F) and central necrotic areas (G).]
necrotic tissues as regions with less blood vessels and low blood flows.\textsuperscript{41} Immediately after i.v. injection, the nanoparticles are transported to perfused regions. Owing to the low interstitial pressure in parts of the tumor, tissue extravasation of nanoparticles is possible. Afterward they can diffuse to necrotic areas where they accumulate. Thus loaded nanoparticles would allow high intratumoral drug concentrations, and the antinecrosis could start from the tumor center. The low interstitial pressure would also explain that no size dependent differences between the accumulation of batch A and B nanoparticles were found within HT29 tumor tissues. A2780 tumor tissues are better supplied with blood (Figure 4 F). Numerous blind ends, occlusions, and wall defects of the tumor blood vessels indicate the renal excretion of the particle accumulation.\textsuperscript{42}

**Ex Vivo Biodistribution Analysis.** The degree of nanoparticle accumulation in different organs and tissues based on ex vivo fluorescence measurements is shown as normalized total and maximum intensities in Table 3. Owing to the linear relationship between exposure time and fluorescence intensity, the Maestro software allows the calculation of exposure-time independent total and maximum intensities. By a previously described calculation method, different nanoparticle batches with consequently varying DiR concentration could be compared.\textsuperscript{25}

Measured total intensities as the sum of all pixels of the respective organ and tissue are shown in the left rows of Table 3. It is visible that highest intensities were found in both organs of the RES, the liver, and the spleen. Batch A and B nanoparticles accumulated in similar amounts in the liver. The smaller size and the higher PLA/PEG ratio of batch A nanoparticles (PLA with a molecular weight of 20 kDa and consequently a higher amount of PEG) compared to batch B had no positive effect to reduce liver accumulation. This might be explained by an optimum size range for both batches and a completely PEG covered surface already in the case of batch B nanoparticles with an PLA/PEG ratio of 20:1 which cannot be improved by higher PEG amounts. However, for batch C nanoparticles with the largest mean size, liver accumulation was clearly highest. The larger fraction of nanoparticles with bigger size (Figure 1) yielded in a nearly double amount of nanoparticles in the liver. This observation is in agreement with other published data where an increased nanoparticle uptake by the RES was found in size ranges between 150 and 300 nm.\textsuperscript{14}

The intensity levels of all other organs and tissues were in the single-digit range. Nearly the same values were obtained for the male and the female mouse group. This underlines the reproducibility of our in vivo studies. The corresponding maximum intensity signals are also given in Table 3 (right rows). The maximum intensities allow a restricted comparison of organs and tissues varying in size although the errors of the measurement especially at the lower limit highly increase. Liver and spleen have comparable optical properties. Therefore, the maximum intensity allows a comparison of the accumulation rate between the bigger liver and the much smaller spleen. The results indicate a slightly decreased liver and an increased spleen uptake for the smaller nanoparticles. The maximum intensity results further confirm that no specific accumulation occurred in the kidneys, the lung, the fat, and the heart. An accumulation of nanoparticles based on higher maximum fluorescence intensities, measured for batch A in the lung and for batch B and C in the heart, could not be confirmed in the fluorescence images as fundamental accumulation. The slightly higher intensity level in the gall bladder and the intestine indicate the renal excretion of the particles, which was visually confirmed earlier.\textsuperscript{25}

**In Vivo and ex Vivo Tumor Accumulation Analysis.** Normalized total and maximum tumor intensities based on in vivo and ex vivo imaging data for all three batches are shown in Figure 4AB. The calculated total intensities of

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**TABLE 3. Normalized Total and Maximum Intensities of Different Mouse Organs and Tissues, Measured ex Vivo**

<table>
<thead>
<tr>
<th>batch</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver</td>
<td>39.8</td>
<td>45.0</td>
<td>34.8</td>
<td>37.4</td>
<td>100.0</td>
<td>88.8</td>
</tr>
<tr>
<td>spleen</td>
<td>8.8</td>
<td>9.3</td>
<td>9.0</td>
<td>0.5</td>
<td>12.4</td>
<td>15.2</td>
</tr>
<tr>
<td>gall bladder</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>intestine</td>
<td>1.5</td>
<td>1.2</td>
<td>0.5</td>
<td>1.2</td>
<td>4.5</td>
<td>5.3</td>
</tr>
<tr>
<td>kidney</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>lung</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>fat</td>
<td>1.5</td>
<td>1.2</td>
<td>0.5</td>
<td>1.2</td>
<td>4.5</td>
<td>5.3</td>
</tr>
<tr>
<td>heart</td>
<td>1.5</td>
<td>1.2</td>
<td>0.5</td>
<td>1.2</td>
<td>4.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

* Data in percent based on three male mice per nanoparticle batch, 24 h after injection. 
* Data in percent based on four female mice per nanoparticle batch, 24 h after injection. 
* Data are based on previously shown results. 

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\textsuperscript{14} Owing to the low interstitial pressure in parts of the tumor, tissue extravasation of nanoparticles is possible. Afterward they can diffuse to necrotic areas where they accumulate. Thus loaded nanoparticles would allow high intratumoral drug concentrations, and the anticancer therapy could start from the tumor center. The low interstitial pressure would also explain that no size dependent differences between the accumulation of batch A and B nanoparticles were found within HT29 tumor tissues. A2780 tumor tissues are better supplied with blood (Figure 4 F). Numerous blind ends, occlusions, and wall defects of the tumor blood vessels indicate the renal excretion of the particle accumulation.\textsuperscript{42}

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**In Vivo and ex Vivo Tumor Accumulation Analysis.** Normalized total and maximum tumor intensities based on in vivo and ex vivo imaging data for all three batches are shown in Figure 4AB. The calculated total intensities of
the A2780 tumors based on in vivo and ex vivo measurements are shown in Figure 4A. It is visible that the total intensity remained constant between 3 and 5 h and was doubled after 24 h. This might be explained by the long circulation time of the nanoparticles. Blood vessels were detectable up to 6 h, still indicating circulating nanoparticles. During the next hours an increasing number of nanoparticles accumulated in the tumor and those still circulating were continuously removed by the RES. The ex vivo signals, measured 24 h after injection are in good agreement with the in vivo obtained total intensities (Figure 4A). The decreased level of the total intensity measured ex vivo is the result of the slightly imprecise tumor definition within the ROI analysis of the in vivo cube file. The degrees of nanoparticle accumulation in A2780 and HT29 tumor tissues based on ex vivo data are shown in Figure 4B,C. To allow comparison between the larger A2780 and the smaller HT29 tumors both the normalized total and the normalized maximum intensities were divided by the respective ex vivo determined tumor volume. It is clearly visible that nearly the same tendencies were obtained for both normalized total and normalized maximum intensities. Lowest tumor accumulation generally was found for batch C nanoparticles. This can be explained by the above-discussed efficient elimination of the particles from the bloodstream by the RES (Table 3). For the A2780 ovarian carcinoma xenograft a strong size dependence of tumor accumulation was found (Figure 4B): Batch A nanoparticles with the smallest size (z-average = 111 nm, D90 = 80 nm) showed ~50-fold higher total intensities compared to the total intensities measured for batch C and ~2-fold compared to that of batch B. Examining the normalized maximum intensity of the A2780 tumor (Figure 4C), even a ~27-fold increase of the intensity between batch C and A was observed. The size difference between both batches A and B of 30 nm based on the z-average values (PCS measurements) and of 70 nm based on the D90 diameter results (AF4/MALLS analysis) resulted in a more than doubled enrichment for the smaller nanoparticles (Figure 4B,C). Within comparable liver accumulations between batch A and B nanoparticles we expected that the smaller size of batch A had a positive tumor-enhancing effect. Owing to high perfusion and vascular permeability of the A2780 ovarian carcinoma, this might be based on the EPR effect which is known to be highly size dependent.

In contrast to the A2780 tumors, the highest accumulation in the HT29 tumor was detected for the medium-sized nanoparticles (batch B, z-average = 141 nm, D90 = 153 nm). Batch A nanoparticles showed comparable but slightly decreased accumulation rates. Batch C (z-average = 166 nm, D90 = 230 nm) appeared to be not suitable for tumor accumulation. The tumor-size-related total and maximum intensity of batch C were 5- to 9-fold reduced compared to the values of the other two batches. Based on the tumor size related calculations nearly the same normalized maximum and total intensity values (Figure 4B,C) indicate for HT29 and A2780 a homogeneous tumor accumulation.

CONCLUSION

In the present study, we investigated the size-dependent in vivo fate of PEG—PLA nanoparticle batches with different but well-defined sizes. Therefore, detailed particle size characterizations prior to the in vivo studies were carried out. We could show that nanoparticle batches with different sizes (z-averages between 111 and 166 nm in our study) within the optimum size range for tumor accumulation21 can be produced. Noninvasive multispectral NIR fluorescence imaging studies allowed nanoparticle detection just after injection up to 48 h. Combining DsRed2 expressing HT29 tumor cells with in vivo fate studies permitted nanoparticle tracking simultaneously next to tumor visualization from in vivo to ex vivo, up to cellular ranges. The confocal microscopic pictures confirmed the ex vivo imaging results where particles were located between fluorescent, DsRed2 expressing cells. In vivo studies with HT29 and A2780 tumor bearing mice showed that nanoparticles accumulated in both tumors. Interestingly, the ex vivo studies furthermore demonstrated that the accumulation pattern mainly differs between both used tumor models. In the HT29 tumor tissue nanoparticles enriched in the tumor center primarily, whereas A2780 showed no centralized nanoparticle accumulation. Furthermore, highest tumor enrichments were found with batch A nanoparticles for the A2780 and comparable accumulations for batch A and B nanoparticles in the case of HT29 tumors. This points to the fact that the accumulation in centralized necrotic fields (HT29) is not as size dependent as it is for vascular permeation (A2780). Ex vivo viscera distribution studies showed distinct differences between the larger particles of batch C compared to those of batches A and B. The increased particle size yielded in high uptake rates by the RES and therewith to very low tumor accumulations. The presented results showed impressively the size-dependent in vivo behavior of produced nanocarriers. Slight differences between the z-averages of ~30 nm (between batch A and B) and of ~20 nm (between batch B and C) with—compared to literature—rather low PDI values (between 0.13 and 0.16) resulted in strongly different in vivo results. The addition of AF4/MALLS as a further particle size measurement method attested that all three nanoparticle batches varied more in size than PCS data would let expect. Whereas D50 values yielded same size intervals as measured within the PCS results (~20 and ~30 nm), D90 results attested size differences of ~70 nm (between batches A and B) and
Supplemental material

(b) Characterization of nanocarriers and their potential usage in cancer therapy

MATERIALS AND METHODS

Materials. 3,6-Dimethyl-1,4-dioxane (α-lactide), poly(ethylene glycol) monomethyl ether (mPEG20000, MW = 2000 Da), stannous 2-ethylhexanoate (>95%), phosphate buffered saline (PBS) buffer solution, and sorbitol were obtained from Sigma Aldrich, Germany. Sucrose was obtained from Merk KGaA, Germany, poloxamer 188 (Pluronic F68) from Riedel-de Haën, Germany, and paraffin as well as formalin (≥35%) from Carl Roth, Germany. The fluorescence dyes 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine iodide (DiR), Dulbecco’s phosphate buffered saline (PBS) and the Vybrant DIO cell-labeling solution (DIO) were purchased from Invitrogen, Germany. RPMI-1640 medium, 1% streptomycin/penicillin solution, and fetal bovine serum (FBS) were provided from PAA, Germany. RPMI-1640 medium, 1% streptomycin/penicillin, 10% fetal bovine serum and 1% dimethyl sulfoxide (DMSO) were purchased from Gibco, Germany. Polystyrene standard nanoparticles were obtained from Duke Scientific, United States (50, 100, 200 nm), and from Beckman Coulter, Germany (300 nm). All other substances and solvents were used as received.

Synthesis and Preparation of PEG–PLA Nanoparticles. The synthesis of the diblock copolymers PEG-PLA80 and PEG-PLA40 followed a previously established procedure. The numbers refer to the molecular weight of the respective polymer block (in kDa). A nanoprecipitation method was used for the nanoparticle preparation as described earlier. In brief, a solution containing different amounts of polymer in 5 mL of chloroform and the NIR dye DiR (for batches A containing different amounts of polymer in 5 mL of chloroform) was dropwise added to 60 mL of an aqueous solution containing 0.25% (w/v) poloxamer 188. Thereafter, the organic solvent was removed by evaporation under stirring until the mixture reached room temperature. The nanoparticles were then collected by centrifugation, washed with purified water, resuspended in 5% sucrose solutions and subsequently freeze-dried.

Physicochemical Nanoparticle Characterization. Fluorescence Spectroscopy. Fluorescence spectra (775–900 nm) of the nanoparticles dispersed in water (nanoparticle concentration 0.23 mg/mL) were recorded using a LS 55 spectrophotometer (PerkinElmer, United States) equipped with a red-sensitive R928 photomultiplier (750 V), following excitation with 705 nm. The measured intensities were subsequently normalized to the particle concentrations.

Photon Correlation Spectroscopy (PCS). For PCS measurements, all three DiR-loaded nanoparticle batches (batch A–C, Table 1) as well as a dye free PEG-PLA40 batch (batch D) were diluted with purified, filtered (0.2 μm) water to reach a nanoparticle concentration of 0.1 mg/mL. The unloaded nanoparticles were furthermore dispersed in purified water containing 10% PBS buffer, 10% FBS and 10% of both, PBS and FBS, respectively. The measurements were carried out with a high performance particle size detector (HPPS, Malvern Instruments, Germany). The samples were measured four times in the backscattering mode (173°) at room temperature (25 °C) with 12–16 runs over 10 s each at a fixed measurement position in the middle of the cuvette. Samples in PBS and FBS were measured after 24 h of storage to detect possible interactions between the nanoparticles themselves or between the nanoparticles and serum components. The mean particle size (z-average) and the PDI (polydispersity index) were determined by the instruments cumulant analysis software (version 4.20), n = 4 for batches A–C and n = 3 for batch D.

Asymmetrical Field Flow Field Fractionation (AFF). Particle size distributions of the nanoparticles were measured by AFF (Eclipse, Wyatt Technology Europe, Germany) coupled with a MALLS detector (DAWN EOS, Wyatt) under the same conditions as used in our previous study. Size distributions were calculated by the Astex software 4.90 (Wyatt) using the particle mode and assuming compact spheres. Characteristic diameters (D10, D50, and D90) were obtained from cumulative size distributions. All three nanoparticle batches (Table 1) were diluted with purified, filtered (0.2 μm) water (concentration, 1 mg/mL), successively injected into both flanks of nude mice. At a size of approximately 1.5 cm3, 100 μL of batch A nanoparticles was injected and time dependent images were grabbed by fluorescence imaging. For detailed size dependent tumor accumulation studies nonfluorescent s.c. xenograft tumors were established in 13 nude mice. A total of 3 × 106 HT29 cells were s.c. injected to the right flank of the mice and 3 × 107 cells of A2780 were injected into the left side. Body weights and tumor sizes were measured at 3 days post-injection. The unloaded nanoparticles (1 mg/mL) were also dispersed in purified water and water supplemented with 10% and 50% FBS, respectively. All samples were measured with purified water (preserved and 0.2 μm filtered) as carrier liquid in triplicate, and results are given as an average with standard deviation. The accuracy of the A4F/MALLS separation system was routinely checked using a mixture of 50, 100, 200, and 300 nm polystyrene standard nanoparticles.

Cell Culture for Confocal Microscopy. Human colon carcinoma cells (HT29) and human ovarian carcinoma cells (A2780) were cultivated as monolayers on round cover glasses with 80,000 HT29 or 85,000 A2780 cells per cm2. Both cell lines were seeded and incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin at 37 °C, 95% humidity, and 5% carbon dioxide. After 24 h the medium was removed and each of three round cover glasses with cells were incubated for 24 h with either unloaded, DiR-loaded nanoparticles (batch A, Table 1), or pure dispersion medium. A second series of each of three round cover glasses with cells were equally handled but stained additionally with DIO (Vybrant standard procedure) to visualize the cell membranes next to nanoparticles. After 24 h of incubation, the medium was removed and the cells were fixed with formalin (2%/v/v) for 20 min at 37 °C. After washing the cells with PBS for three times, they were immediately imaged in a confocal microscope.

Confocal Microscopic Analysis. The LSM 710, a flexible confocal microscope (Zeiss, Germany), allows studying the cellular uptake of nanoparticles as well as of ex vivo excised tissues. The microscope was equipped with the Plan APO 63× oil immersion objectives. The DiR was excited with a 633 nm laser. Emitted fluorescence light was detected from 650 nm to the upper detection limit. The excitation of DsRed2, a red fluorescent protein, was carried out using the 514 nm laser. The 458 nm laser was used to excite DIO. Images were acquired in a sequential scan mode and processed using the ZEN software (Zeiss, Germany). To image the ex vivo samples the tumor tissue was excised and cut into small panels (thickness approximately 0.5 mm) by a raker plate. Afterward the tissue was slightly pressed between two cover glasses and immediately viewed with the LSM 710.

Animal Models and Nanoparticle Injection. Nanoparticle accumulation studies were performed in male NMRI-nu (nu/nu) mice with janssen with prior approval. All experiments complied with the standards for use of animal subjects as stated in the guideline from the animal care and use committee of Saxony Anhalt. Aqueous nanoparticle dispersions (≤0.1 mg nanoparticles per milliliter) were prepared by redispersing adequate amounts of the freeze-dried nanoparticles (stabilized with 5% sucrose) in purified water containing 2.25% sorbitol to adjust tonicity. A 100 μL portion of the dispersion was slowly i.v. injected into the tail vein of non-narcotized mice using a 30 Gauge needle. During imaging the mice were narcotized and protected for cooling out.

Xenograft Tumor Model and Application. HT29 and A2780 cells were maintained as monolayer cultures as given above. DsRed2 expressing HT29 cells were generated by lentiviral transduction according to the protocol described previously. After growing, 3 × 106 DsRed2 HT29 cells were subcutaneously injected into both flanks of nude mice. At a size of approximately 1.5 cm3, 100 μL of batch A nanoparticles was injected and time dependent images were grabbed by fluorescence imaging.

For detailed size dependent tumor accumulation studies nonfluorescent s.c. xenograft tumors were established in 13 nude mice. A total of 3 × 106 HT29 cells were s.c. injected to the right flank of the mice and 3 × 107 cells of A2780 were injected into the left side. Body weights and tumor sizes were measured at 3 days post-injection.
Supplemental material

(b) Characterization of nanocarriers and their potential usage in cancer therapy

Twice a week. After the A2780 achieved a maximum tumor size of about 2 cm³, the mice were separated into three groups (three mice each) and care was taken for a preferably homogeneous tumor size distribution. One untreated mouse was used in vivo control and for further ex vivo microscopy studies. A 100 µL portion of each nanoparticle batch was injected in all three mice of the respective group: A, B, and C (named like the nanoparticle batch as in Table 1). The mice were imaged 24 h after injection and sacrificed, and the respective tumors were excised. The tumor was exactly determined with a caliper, and the tumor volume was calculated using the tumor dimensions of all 3 room directions assuming an elliptic tumor shape. All excised tumors were imaged as complete tissue and after slicing into 2 pieces.

In Vivo and In Vivo Tumor Imaging. In vivo and ex vivo fluorescence imaging experiments were preformed using the Maestro in vivo fluorescence imaging system (Cambridge Research and Instrumentation, United States). The green filter set (503–555 nm excitation and 580 nm long-pass emission filters) was used to detect DsRed2 tumor cells. For nanoparticle imaging the NIR filter set with a 710–760 nm excitation and an 800 nm long-pass emission filter was used as described in our previous study.78 To prevent fluorescence interferences between nanoparticle accumulations in the tumor and in the RES, the area of the liver was masked with a black plastic plate in selected imaging experiments. Recordings (cubes) were analyzed using in vitro DiR and cellular DsRed2 spectra as references, and the signal from an untreated mouse was set as background. The cubes were unmixed and segmented using these respective 2 or 3 spectra and saved as monochrome images. On the basis of these images, RGB (red green blue) pictures were generated allocating a respective color (DsRed2 in green and DiR in red) to the spectra. The generated grayscale images were also intensity-weighted illustrated.26

For ex vivo analysis the mice were sacrificed using carbon dioxide 24 h after injection of the nanoparticles (48 h in the case of DiRred expressing HT29 experiments). The excised tumors and organs (liver and spleen) were imaged with the Maestro in vivo/mag system using the same parameters as in the in vivo study. To ensure reproducible conditions the organs (liver and spleen) were placed in a 24 holes well plate. An area of interest (ROI) in the size of the well plate hole was generated. Total and maximum intensities of all pixels in the ROI were measured and correlated to the respective exposure times by the software. To exclude intensity variations due to different initial dye concentrations and intensities between the nanoparticle batches, a previously described correction method was applied.24 The total signal (correlated to the exposure time) was divided through the in vivo emission peak maximum which was determined prior to the in vivo measurements. The ex vivo total and maximum intensity signals of the nanoparticle accumulation in the tumor tissues were furthermore divided through the ex vivo determined tumor size (volume of an ellipsoid, based on three room directions). All graphs are normalized to 100% related to the highest result. Excised tumors were fixed with 4% formalin for 1 week and afterward embedded in paraffin. Resulted blocks were sliced (4 nm), dewaxed, and stained with hematoxylin and eosin (H&E). Light microscopic images of the stained tissues were obtained using a Zeiss Axioslab microscope (Zeiss, Germany).

Acknowledgment. We thank Jürgen Vogel and Marcus Niepel for supporting the confocal microscopic measurements. Mr. Jorg Tesmar is acknowledged for the discussions during polymer synthesis and nanoparticle preparation. We thank also Martina Hennicke and Constanze Gottschalk for the animal care. The in vivo studies were partly supported by the Federal State of Saxonia Anhalt (FKZ 36464/A907) and the confocal microscopy studies by the Deutsche Forschungs-gemeinschaft (LSM: INST 271/250-1).

REFERENCES AND NOTES


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Accumulation of nanocarriers in the ovary: A neglected toxicity risk?

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A B S T R A C T
Several nanocarrier systems are frequently used in modern pharmaceutical therapies. Within this study a potential toxicity risk of all nanoscaled drug delivery systems was found. An accumulation of several structurally different nanocarriers but not of soluble polymers was detected in rodent ovaries after intravenous (i.v.) administration. Studies in different mouse species and Wistar rats were conducted and a high local accumulation of nanoparticles, nanocapsules and nanoscaled lipid emulsion in specific locations of the ovaries was found in all animals. We characterised the enrichment by in vivo and ex vivo multispectral fluorescence imaging and confocal laser scanning microscopy. The findings of this study emphasise the role of early and comprehensive in vivo studies in pharmaceutical research. Nanocarrier accumulation in the ovaries may also comprise an important toxicity issue in humans but the results might as well open a new field of targeted ovarian therapies.

1. Introduction

Until now, a possible accumulation in ovarian tissue has not been investigated in biodistribution studies of nanocarriers. Therefore, extensive in vivo and ex vivo studies were performed within this study to examine this in detail. In our present study we measured the accumulation of differently composed nanoscaled drug delivery systems in the ovaries of rodents. To investigate the correlation of carrier size and ovary accumulation, detailed size distributions of all systems were measured by asymmetrical flow-field-flow fractionation (AF4). The accumulation in ovaries was characterized in vivo and ex vivo in different mouse strains and Wistar rats using non-invasive fluorescence imaging and confocal laser scanning microscopy (CLSM). Fluorescence imaging is a powerful technique to investigate the body fate of drug delivery systems noninvasively over several hours up to days or months in the same organism. The fluorescence imaging studies were carried out using consequently near infrared (NIR) fluorescent dyes that provided information also from deep tissues as well as from tissues which are highly supplied with blood. Those dyes were either covalently stable bound via amide bonds (polymers) or physically entrapped into the carriers (nanoparticles, nanocapsules and nanoscaled lipid emulsion).

In total, we were able to prove the ovary accumulation of 5 different nanocarrier batches with diameters between 45 and 350 nm.

2. Materials and methods

2.1. Materials

3,6-dimethyl-1,4-dioxan (DL-lactide), poly(ethylene glycol) mono-methyl ether (mPEG2000, MW= 2000 Da), stannous 2-ethylhexanoate...
Supplemental material

(b) Characterization of nanocarriers and their potential usage in cancer therapy

 (>95%), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), ethylene diamine, tolueine sulfonil chloride and sorbitol were obtained from Sigma Aldrich, Germany and poloxamer 188 (Pluronic F68) from Riedel-de Haën, Germany. Sucrose was obtained from Merck KGaA, Germany and PEG 5 kDa) and the Lipofundin 20 N were purchased from Invitrogen, Germany. The fluorescence dye 1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine iodide (DiR) was purchased from Invitrogen, Germany. The fluorescence dye IRRDye 800CW was obtained from Li-COR, US. The diblock copolymer PLGA-PEG 45–5 (PLGA 50:50 45 kDa and PEG 5 kDa) and the Lipiodol 20 N were purchased from Boehringer Ingelheim, Germany and Lipiodol (Lipiodol Ultra fluid) from Guerbet GmbH, Germany. Hydroxyethyl starch 200/0,5 (batch HES) and dextran 500 (batch DEX) were kindly provided by Serumwerke Bernburg AG, Germany. Formaldehyde was purchased from Carl Roth GmbH, Germany. All other substances and solvents were used as received.

2.2. Preparation of nanoparticles

As described earlier [14] a nanoprecipitation method was used for the preparation of 3 NIR fluorescent nanoparticle batches, differing in size (batch NP1–NP3, Table 1). 37.5 mg (batch NP1) PEG5PLGA50 or PEG5PLGA100 (50 mg for batch NP2 or 100 mg for batch NP3), the numbers refer to the molecular weight in kDa, were dissolved in 5 mL chloroform. Referring to the polymer, 0.5% (batch NP1) or 1.0% (batch NP2 and NP3), of the NIR emitting fluorescence dye 1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine iodide (DiR) were added. To form solid nanoparticles, the solution was dropped into 40 mL of an aqueous solution containing 0.25% Pluronic F68. After removal of organic solvent by evaporation, the nanoparticles were collected by centrifugation and washed with purified water. Subsequently 5% sucrose was added and the dispersion was freeze-dried.

2.3. Preparation of nanocapsules

Nanocapsules were prepared by interfacial polymer deposition after solvent displacement (batch NC, Table 1). 20 mg PLGA-PEG 45–5 (PLGA (45 kDa) and PEG (5 kDa), 50:50) and 50 μL Lipidol, loaded with 100 μg DiR (batch NC) or with 100 μg DiI (batch NC–DiI, Table 1) were dissolved in 2 mL acetone. The solution was dropwise injected into 4 mL water under stirring. The acetone was evaporated (under reduced pressure at 30 °C) and all samples were centrifuged (for 15 min at 4000 rpm) using a Minispin from Eppendorf, Germany. The supernatant containing dye loaded nanocapsules was 2 mL.

2.4. Dye loading of a nanosized lipid emulsion

For comparison also a commercially available lipid emulsion (Lipofundin 20 N) was loaded with DiR. An ethanolic DiR solution (100 μg/mL) was dropwise added under continuous stirring (batch LE, Table 1) to get a final dye loading with the same fluorescence intensity as it was achieved for the nanocapsule batch.

2.5. Preparation of soluble polymers

Hydroxyethyl starch (batch HES, Table 1) and dextran (batch DEX, Table 1) were amino-modified using ethylene diamine and covalently coupled with the NIR-fluorescent dye IRR800CW. The amino groups were introduced to the molecules based on a previously described method [16]. Toluene sulfonyl chloride (0.5 g) was slowly added to a DMF solution (30 mL) of 1 g polymer and 1 mL triethyl amine. The mixture was cooled on ice and reacted for 2 h in the dark. After precipitating in acetone, the polymers were dialysed against water (3.5 kDa membrane from Spectrum Labs, United States) and subsequently freeze dried. The tosyl-modified polymers (0.25 g) were reacted with ethylene diamine (1.5 g) in DMF (50 mL) and borax buffer (pH 10) for 20 h. Resulting polymers were precipitated in methanol and isopropanol (1:1), afterwards several times dialysed against water and subsequently lyophilised. The amino-modified polymers (100 mg) were dissolved in water (50 mL), reacted with 0.5 mg IRRDye 800CW in the dark for 3 h and once again dialysed against water and afterwards lyophilised.

Table 1

<table>
<thead>
<tr>
<th>Batch</th>
<th>Polymer/ carrier</th>
<th>Size</th>
<th>Animals per batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymeric (flexible)</td>
<td>Batch HES</td>
<td>HES 200</td>
<td>30 nm (Dw)⁴</td>
</tr>
<tr>
<td></td>
<td>Batch DEX</td>
<td>Dextran 500</td>
<td>35 nm (Dw)⁴</td>
</tr>
<tr>
<td>Nanoparticles (solid)</td>
<td>Batch NP1</td>
<td>PLA50PEG50</td>
<td>45 nm (D50)⁵</td>
</tr>
<tr>
<td></td>
<td>Batch NP2</td>
<td>PLA50PEG50</td>
<td>65 nm (D50)⁵</td>
</tr>
<tr>
<td></td>
<td>Batch NP3</td>
<td>PLA50PEG50</td>
<td>100 nm (D50)⁵</td>
</tr>
<tr>
<td>Nanocapsules (solid shell)</td>
<td>Batch NC</td>
<td>PLA20PEG20 Lipiodol</td>
<td>55 nm (D50)⁵</td>
</tr>
<tr>
<td></td>
<td>Batch NC-DiI⁶</td>
<td>PLA20PEG20 Lipiodol</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanosized lipid emulsion droplets</td>
<td>Batch LE</td>
<td>Lipofundin 20 N</td>
<td>350 nm (Dw)⁸</td>
</tr>
</tbody>
</table>

⁴ Dw means the weight-average mean square diameter, measured by AF4/MALLS.
⁵ D50 means the mass weighted distribution median diameter, measured by AF4/MALLS.
⁶ Batch NC-DiI was only used for additional confocal microscopy studies. It was injected into 2 SKH1–Hrhr mice (♀).
⁷ Batch LE was used for additional confocal microscopy studies. It was injected into 2 SKH1–Hrhr mice (♀).

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2.6. Particle size measurement

All nanocarrier batches were analysed by AF4 based on a method previously described [14]. The fractionation system (AF4/MALLS) was composed of an Eclipse AF4 (Wyatt, Germany) which was coupled with a multi-angle laser light scattering (MALLS) detector (DAWN EOS, Wyatt). Particles were separated in a trapezoidal-shaped channel (length 265 mm, largest width 21 mm, height 350 μm) in dependence on their size and shape by applying appropriate cross flow rates. A 5 kDa membrane (regenerated cellulose or polyethersulfone (MWCO 5 kDa, Microdyn-Nadir, Germany) was used as accumulation wall. Bi-distilled water served as carrier liquid (preserved with 0.02% sodium azide and filtered through 0.1 μm pore sized filter). A volume of 100 μL of the nanocarrier dispersions (1 mg/mL for batch NP1–NP3, for batch NC and for batch HES and batch DEX, 0.1 mg/mL for batch LE) were injected into a channel (flow, 2 ml/min). Samples were eluted with a constant (1 ml/min) and decreasing cross-flow (from 2 to 0.5 ml/min within 5 min and from 0.5 to 0 ml/min within 35 min). The particle sizes were calculated based on the light scattering signal. For the batches NP1–NP3, NC and LE the particle mode was used for size evaluations (assuming compact spheres), whereas for the polymers the molecular weight mode was used. The Astra software 4.90 (Wyatt, Germany) allowed determining the corresponding mean diameters (D50) from the mass weighted distributions [14]. Polymer sizes were calculated based on weight-average mean square diameters (Dw) using RI detector signal. All dispersions were measured in triplicate, and results are given as average in Table 1.

2.7. Animal handling

All experiments complied with the standards for use of animal subjects as stated in the guideline from the animal care- and use-committee of Saxony Anhalt. As listed in Table 1 in vivo studies were performed in pubescent female (batches NP1–NP3, NC and LE, all in quadruplicate, batches HEX and DEX in triplicate and batch NC–DiI in duplicate) and male (batches NP1–NP3, all in triplicate), nude SKH-1/Hr μm mice (age: 2–12 months). Further in vivo studies were performed in female BALB/c mice (batch NC, n = 3, age: 6 months) and in female Wistar rats (batch NC, n = 3, age: 12 months). The animals were housed under controlled conditions (12 h light/dark schedule, 24 °C). Respective samples were isonitised with sorbitol and slowly i.v. injected into the tail vein (100 μL for mice and 1 mL for rats, in a concentration of 8 mg/mL for the nanoparticle and nanocapsule batches and 10 mg/mL for the polymers). For in vivo fluorescence imaging, the mice were anesthetised using a mixture of isoflurane and oxygen with an initial flow of 4% isoflurane (3.0 L/min oxygen) and a steady state flow of 1.8% isoflurane (1.5 L/min oxygen). The mice were placed on a 35 °C temperature-controlled heating plate to prevent a decrease of body temperature. For ex vivo analysis mice and rats were sacrificed using carbon dioxide 24 h after injection.

2.8. Fluorescence imaging

In vivo and ex vivo fluorescence imaging experiments were performed using the Maestro in vivo fluorescence imaging system (CRi, now Caliper Life Sciences, United States) and the Maestro software (version 2.10) [14,15]. The NIR filter set (710–760 nm excitation and 800 nm long pass emission filter) was used for the detection of both NIR dyes, the DiR and the IRDye 800CW. The measurement files (cubes) were grabbed in the spectral range between 780 and 950 nm using auto exposure times. The tuneable filter was automatically stepped in 10 nm increments and intensity weighted greyscale images were acquired at each step.

The single spectral series were unmixed from the recorded cubes using reference spectra of DiR or IRDye 800CW and the background sig- nal of an untreated mouse that was measured under same conditions. During the unmixing process, each pixel was intensity weighted allocat- ed to the respective reference spectra. The resulted greyscale images of the respective fluorescence signals were used for further analysis or to generate jet color images [14]. Those have an intensity weighted incre- mental color profile. Pixels with maximum intensities are set to dark red and pixels with no fluorescence to dark blue/black. In between there is a graduation from red to orange, yellow, light and dark blue.

For ex vivo analysis, the mice and rats were sacrificed 24 h after in- jection. The uteri with ovaries (female mice and rats) or seminal ves- icles, testes (male mice) were excised and imaged with highest possible magnification on a plastic plate. The spectral series were unmixed from the image cubes as described above. Ovaries and uterus from untreated mice served as negative controls.

For batches NP1–NP3, the accumulation in the ovaries was also com- pared with the distribution of the nanoparticles in other organs and tis- sues. Therefore, the intestine, fat, liver, gall bladder, lung, spleen, kidney and heart were placed next to uterus with ovaries into 9 separate holes of a 24 holes well-plate. This allowed reproducible measurement condi- tions for the organs and tissues of all mice. The plate was imaged with the Maestro in vivo imaging system. To detect also minor nanocarrier accumulations, the measurements were repeated after masking the liver with a black plastic plate. Organs from untreated mice served as negative controls. A region of interest (ROI) in the size of the well-plate hole (1.9 cm²) was generated to extract total and maximum inten- sities of the organs and tissues. The software correlated them to the respective exposure times. To exclude intensity variations due to different initial dye concentrations and intensities between the 3 differ- ent nanoparticle batches (NP1–NP3), a previously described correction method was applied [14]: For this, the total signal (correlated to the ex- posure time) was divided by the in vitro emission peak maximum which was determined prior to the in vivo measurements. All values were normalised to 100% referring to the highest result.

2.9. Confocal laser scanning microscopy (CLSM)

CLSM experiments were carried out using a LSM 710 (Zeiss, Ger- many). Dyes were excited using an Ar-Laser for DiI (514 nm) and DiO (488 nm) and a HeNe-Laser for DiR (633 nm). Samples were pre- pared from just extracted ovaries that were cut into small slices of app. 0.5 mm using a razor blade. Afterwards the slices were trans- ferred to an object slide. Further, frozen sections of extracted ovaries were stained with DiO to visualise the cellular membranes. The 40x or the 63x Plan Apo oil immersion objectives were used for microscopy. For comparison, also a pure solution of DiI stained nanocapsules was imaged under the same conditions like the sliced ovaries. To obtain a 3-dimensional impression of the nanoparticles distribution in the mi- crosopic ovary structures Z-stacks were applied using 80 μm steps. All images were grabbed and processed with scale bars using the soft- ware ZEN 2008 (version 1.0.3).

2.10. Light microscopy

An AxioLab Microscope from Carl Zeiss Microlmaging (Germany) was used for light microscopy of ovarian tissue. Excised ovaries were fixed with 4% paraformaldehyde and embedded in paraffin. Thin slices (4 μm) were cut using a microtome and transferred to ob- ject slides. The slices were dewaxed and stained with Haematoxylin and Eosin. Images were grabbed and processed using the Zeiss Axio- lab software.

3. Results

3.1. Particle size measurements

For particle size measurements AF4 was combined with MALLS. This allowed accurate size evaluation of all batches due to sample
separation prior size determination. The characteristic D50 (for nanocarriers) and D90 (for polymers) are shown in Table 1. All used nanocarriers were within the size range between 45 and 350 nm, whereas diameters of 30 and 35 nm were measured for the flexible polymers with the covalently bound NIR dye. The lipid nanoemulsion was rather broad distributed. All nanoparticle batches were narrow distributed [15]. Based on the determined D50 values of 45 nm (batch NP1), 65 nm (batch NP2) and 100 nm (batch NP3) it is possible to draw conclusions about the size dependent accumulation of nanocarriers in rodent ovaries.

3.2. In vivo and ex vivo fluorescence imaging studies

Within this study, the ovary accumulation of 7 differently composed nanoscaled drug delivery systems (Table 1) was investigated using the Maestro in vivo imaging system. For our study we selected NIR fluorescent dyes, as they can be detected also in deep tissues [17]. Generally, fluorescent dyes can be loaded to the carrier systems by incorporation or by covalent attachment. As incorporation of the fluorescent dye does normally not affect the surface characteristics of the nanocarriers, this method was used for the nanoparticle, nanocapsule and lipid nanoemulsion batches. In case of soluble polymers (batches HES and DEX) the fluorescent dye was covalently coupled to the polymer. For a physical entrapment, a very hydrophobic dye is required which will not diffuse out of the drug delivery system until the carrier is degraded. Therefore, dialkylcarbocyanine dyes (DiR and DiI) were chosen. DiR is a very lipophilic NIR fluorescence dye that has already been used in several in vivo studies to track nanocarriers by fluorescence imaging noninvasively [18,19].

A distinct diffusion of the dye out of the nanocarriers into the blood can be excluded due to the very high partition coefficient of the utilised dye. Reported log P values of dialkylcarbocyanine dyes are between 17 [20] and 20 [21]. The release of those dyes from lipophilic nanocarriers is reported to be less than 4% within the first week [22,23].

In this study (Table 1) DiR was incorporated into 3 batches of different sized PEG-PLA nanoparticles (batch NP1: PEG2PLA20, batch NP2 and NP3: PEG2PLA40), 1 batch of oil loaded PEG5PLGA45 nanocapsules (batch NC) and 1 batch of a common lipid emulsion (Lipofundin 20 N, batch LE). Due to the fact that a physical incorporation is not possible for soluble hydrophilic polymers, the 2 polymer batches HES and DEX were successfully amino-modified and covalently stable labelled with the amine reactive NIR fluorescent dye IR 800CW (NHS-ester).

The long-term in vivo fate was investigated over about 3 weeks in nude female mice (SKH1-Hr). Detailed in vivo distribution studies as Fig. 1. In vivo and ex vivo fluorescence images of accumulated nanocarriers. a, Time dependent lateral optical images of a nude mouse after i.v. nanoparticle injection (batch NP2) presented as an intensity weighted visualization of the extracted fluorescence dye signal (jet color). Areas with highest dye intensities are red and those with lowest dye concentrations are blue. The red arrow marks the liver, the yellow one the spleen and the white one the ovaries. b, Intensity weighted jet color images of the dorsal side from the same mouse as shown in a (arrows point to ovaries). The long term enrichment of the carrier system in the ovaries is visible. c, Ex vivo jet color images of the excised uterus with ovaries 24 h after nanocarrier injection. While the uterus is not or only slightly fluorescent, high fluorescence intensities were detected in the ovaries. d, Ex vivo images of excised ovaries (original photograph (from left to right), greyscale image of the extracted dye signal and corresponding jet color image for a better visualization of the intensity allocation). 24 h after batch NP3 administration (upper row), negative control (lower row). e, Greyscale and corresponding jet color image of the sliced ovary in the highest possible magnification. The fluorescence in the ovaries is concentrated in highly fluorescent spots (marked by arrow).
well as size dependent tumor accumulation studies of used nanoparticle batches NP1–NP3 have been discussed previously [14,15]. The resulting in vivo images are exemplarily shown for medium sized batch NP2 (PEG5PLGA45) nanoparticles in Fig. 1a and b.

An accumulation of the nanoparticles in the organs of the RES was already observed after 2 h. One day after injection, the fluorescence intensity in the RES reached maximum values and decreased continuously thereafter. Additionally, an accumulation of the nanoparticles in the ovaries (Fig. 1a and b marked by white arrows) was detectable already 2 h after administration. The fluorescence intensity from the ovaries further increased within 24 h and remained constant at a high level over several days. Even 25 days after injection a bright fluorescence signal could be detected noninvasively in the area of the ovaries in vivo.

To confirm the ovarian accumulation of nanoparticles, ex vivo experiments with all 3 nanoparticle batches were conducted. For that purpose the uteri with ovaries were excised 24 h after i.v. injection. Representative ex vivo images are presented in Fig. 1c. As it is visible exemplarily in the first 3 images, strong fluorescence intensities were detected in the ovaries of all 12 mice that were treated with nanoparticles (batch NP1–NP3). To evaluate if this observation is not related to the type of nanocarrier e.g. due to a characteristic property of the polymer surface, the experiment was repeated with 2 further nanoscaled formulations (batch NC and batch LE) with a completely different composition. Representative images are shown in Fig. 1c. As it is clearly visible in all images, the uterus itself was low or even not fluorescent. However, the ovaries of all 20 mice were highly fluorescent independently of the size and the surface properties of the administered nanocarrier batches.

To exclude the possibility of measurement artefacts, the ovaries of treated (batch NP2) and untreated mice were measured simultaneously (Fig. 1d). The resulting images of the isolated DRI signal indicate a high nanocarrier accumulation in fluorescent spots whereas no fluorescence at all was found in the control tissues. Magnified images of sliced ovaries, shown in Fig. 1c, confirmed the local and punctual enrichment. To eliminate the possibility of a species specific accumulation in SKH1-Hr mice, in vivo and ex vivo studies with oil loaded PEG5PLGA45 nanocapsules were repeated in 3 female BALB/c mice and 3 Wistar rats. Comparable accumulation results were found in all of the experiments.

An original photograph of the excised rat ovary in comparison to the corresponding intensity weighted jet color image of the same part is shown in Fig. 2a. Due to the increased size of the rat ovary compared to the mouse ones more details are distinguishable. Also in the ovaries of the Wistar rats, local accumulations of the nanoparticles in varying intensities and tissue depths were detectable (marked by arrows in Fig. 2a). The direct comparison of both images also shows that some ball like structures visible in the original photograph are not fluorescent, whereas others are highly fluorescent. This fact indicates that the nanocarrier accumulations might depend on the progress stage within the ovarian cycles.

### 3.3. Confocal microscopy studies

To characterise the local areas of highest fluorescence intensities in more detail, confocal microscopy studies were conducted. Z-stack CLSM images (80 μm steps) confirmed the accumulation in round, ball like tissue structures with an average diameter of approximately 200–300 μm (Fig. 2f). No fluorescence signals were detected in the area of nuclei-like structures (Fig. 2b). However, by CLSM highly fluorescent spots in the ovarian tissue (Fig. 2c, top image) were asignable to the injected nanocarrier dispersion (lower image). In combination with the in vivo results presented in Fig. 1a and b, where fluorescence in the ovaries was detectable for more than 25 days a local long term release of incorporated dyes or drugs from the nanocarriers can be expected. Ex vivo studies were extended by frozen section sliding of excised tissues. In Fig. 2d, accumulated nanoparticles (red) are visible next to cells were the cellular membrane was stained with Dio (green). Fig. 2e shows a stained light microscopic picture of a growing follicle from mouse ovarian tissue.
3.4. Size dependent ex vivo fluorescence imaging analysis

To investigate a potential size dependent accumulation of nanocarriers in the ovaries ex vivo studies of 3 nanoparticle batches (batch NP1–NP3), varying in particle sizes (Table 1) were performed. Nine organs of each mouse (known for nanocarrier accumulation or excretion) were placed, each into a separate hole of a 24 holes well-plate (Fig. 3a). All organs in the well-plate were imaged simultaneously. The well plate allowed the arranging of the tissues and organs of each mouse in the same position for all fluorescence imaging measurements and thus ensured reproducible measurement conditions. The intensity weighted DiR signal as a jet color images are shown in Fig. 3b–d. Organs from untreated mice were imaged as an control. Accumulations in the liver (dark red) as well as in small parts of the ovary (marked by arrow) are visible (Fig. 3c). After covering the liver with a black plastic plate, also weak fluorescence signals from intestine and spleen were detectable (Fig. 3d).

Based on the measured DiR intensity signals, normalised maximum and total intensities of all excised organs were calculated. Corresponding graphs are displayed exemplarily for batch NP3 in Fig. 3e and f. The maximum fluorescence intensity, measured from local spots of the ovaries, was as high as it was detected in the liver, which is the major organ for nanocarrier elimination within the RES. Related to that, the spleen and the gall bladder showed half maximum intensities. All other organs and tissues had intensities near the detection limit. The maximum intensity values allow a comparison of accumulations in organs differing in size. A high enrichment situated in a local part of a tissue for example leads to high maximum intensity values, but the total signal of the same sample, as the sum of all pixels, would only be slightly increased. Thus, the normalised maximum intensities yielded in divergent results compared to the normalised maximum intensities. The calculated total signal of the ovary with the uterus was below 10%, comparable to the much larger intestine. The difference between the total and the maximum signal (10 fold compared to liver results) underline the inhomogeneous and local nanoparticle accumulation in specific regions of the ovary as already above discussed for the ex vivo images.

As shown by the maximum intensities in Fig. 3g, nanoparticles from all 3 batches (NP1–NP3) accumulated in the ovaries. This corresponds to the analysis of the total intensities (Fig. 3h). Compared to male tissues (seminal vesicles and testes), the nanoparticle accumulation in the ovaries was reflected by a 20 fold higher maximum intensity and a 200 fold higher total intensity.

Normalized maximum intensities of batch NP1 and NP2 between 10 and 30% in comparison to 100% for batch NP3 indicate that bigger particles accumulated more than smaller ones, although the standard deviation is rather high here. A higher accumulation was also found by analyzing the total fluorescence intensities. Further investigations using small (<35 nm diameter) water soluble polymers (batch HES and batch DEX) were conducted to investigate whether there is a minimum size required for the accumulation of nanocarriers or not. For this purpose, a dose of 1 mg of each fluorescent labelled polymer in 100 µL isotonic sorbitol solution was injected to the tail vein of 3 female mice (SKH-1-Hr112). No specific accumulation in mouse ovaries

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**Fig. 3.** Ex vivo fluorescence images and analysis of different PEG-PLA nanoparticle batches (NP1–NP3). a–d. Original photograph (a) and jet color images of excised mouse organs from an untreated (b, negative control) and a treated (c and d) mouse (24 h after i.v. injection of batch NP3). For reproducible measurements, these were placed in the following order into 9 holes of a 24-well plate (related to b): intestine, fat, uterus with ovary, liver, gall bladder, lung, spleen, kidney and heart. Jet color images grabbed with 800 ms (c) and 1200 ms (d) exposure time. Both show the accumulation of nanoparticles in the RES and in the ovary (marked by white arrow). e and f. Normalized maximum (e) and total (f) fluorescence intensity graphs of organs, shown in c (n=4), g and h, normalized maximum (g) and total (h) fluorescence intensity graphs of female and male genital tissues of the nanocarrier batches NP1–NP3 (female: n=4, male: n=3).
4. Discussion

An accumulation of nanocarriers in the ovaries is mentioned in a small number of other publications based on in vivo and ex vivo studies with different kinds of nanoparticles and liposomes [24–30]. However, in all of them, the accumulation was neither investigated in detail nor thoroughly discussed. In these publications an ovary accumulation is (I) either visible in the presented pictures or (II) it can be derived from the presented data or (III) is briefly described. The reason, why the accumulation in rodent ovaries may not have attracted attention yet might be the fact that many research groups tend to use male mice. Another reason could be that the accumulation occurs only in certain ovaries, e.g. female mice. Furthermore, no accumulation in mouse ovaries was reported in a recent study of Daou et al. with very small nanoparticles (diameters below 50 nm) [31]. This might be related to the small size of the particles, which would correlate with our results, or to the mice age between 5 and 6 weeks, an age at which they were probably not yet pubescent. Another potential reason for the scarcity of published data on ovarian accumulation could be the observation that the nanocarrier accumulation appears to be restricted only to local parts of the ovaries. The overall amount of fluorescence signal from the ovaries was rather small (Fig. 3f) and not higher compared to other organs and tissues where no nanoparticles accumulated. Commonly, only the average amounts of the tissues or the percentages per administered dosages are compared in in vivo studies. Accumulations in the ovaries might thus often remain unnoticed. Few studies showed that an accumulation of nanoparticles in ovaries is detectable when the accumulation is calculated in μg per tissue weight (g) but not, when it is calculated as the percentage of the dose [26,30]. Anyhow, two fluorescence imaging studies of hybrid- and of lipid-nanoparticles also reported an accumulation [24,25]. Perez-Soler et al. even found liposome accumulations in the ovaries after subcutaneous injection [27]. Harrington et al. also detected a low but significant accumulation of pegylated, radiola beled liposomes in the uterus and ovaries while non-pegylated liposomes were eliminated nearly completely within the first hour [28]. This short circulation time was probably not sufficient to achieve accumulations. Based on the size and structures of the accumulation areas from the ex vivo Maestro and CLSM images of our study, an enrichment in tertiary vesicular follicles might be possible but an accumulation in cells of the corpus luteum seem even more likely.

The corpus luteum is formed from the wall of the ruptured follicles after the ovulation. It is responsible for the production of progesterone which is a key factor during the pregnancy. Progesterone is in high concentrations needed shortly after the ovulation. Thus the ovarian corpus luteum grows and vascularizes extremely fast. Finally, the corpus luteum is typically very large in relation to the size of the ovary. It has been described that the rates of tissue growth and angiogenesis in the corpus luteum rival those of fast growing tumors [32]. They found accumulated nanoparticles in blood vessels around large ovarian follicles and in just formed corpora lutea but not inside follicles and only to a low degree in smaller, regressing corpora lutea [29].

Although the detailed mechanism still has to be enlightened, we could clearly prove specific nanocarrier accumulation in rodent ovaries. This effect might bear a potential toxicity risk if incorporated drugs are locally released in the ovaries. Although, the overall amount was rather small in our study, this effect should be considered and further investigated in future drug delivery studies.

5. Conclusion

By use of in vivo fluorescence imaging, we detected the accumulation of nanoparticles, nanocapsules and nanosized lipid emulsions in specific locations in the rodent ovaries. This effect was further characterised by ex vivo fluorescence imaging and CLSM. The investigated nanocarrier systems were commonly different from each other, including multiple excipients, carrier sizes and surfaces. Based on the extensive in vivo and ex vivo studies it was found that the enrichment seems to be size dependent: whereas, polymers ≤ 35 nm diameter were not accumulated, all tested nanocarrier batches with diameters between 45 and 350 nm highly accumulated in the ovaries. A comparison of 3 nanoparticle batches varying in size led to the conclusion that bigger particles seemed to be more accumulated than smaller ones although this is based on a limited number of experiments. Finally it has to be noted that the accumulation of the nanocarriers in the ovaries does not necessarily need to result in a risk for the widespread use of nanoscaled carrier systems in medicine. Especially due to the fact that the accumulation is limited to special regions in the ovaries, the toxic risk for humans might be rather low. However, this effect needs to be further investigated, particularly also in other species to elucidate the mechanism of accumulation. But also the chances of these results for a new ovarian targeted therapy should be taken into consideration. Our results strongly emphasise the relevance of early explorative in vivo studies in the development of drug delivery systems using sensitive analytical imaging techniques, like fluorescence imaging using NIR fluorescence dyes.

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References

Supplemental material

(b) Characterization of nanocarriers and their potential usage in cancer therapy
Non-invasive in vivo characterization of microclimate pH inside in situ forming PLGA implants using multispectral fluorescence imaging

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Abstract

The pH inside drug delivery systems directly influences the physical and chemical behavior of its ingredients especially their stability and solubility. Therewith the release performance of the systems and the pharmacological effect is affected. Thus the determination of the microclimate pH (μpH) inside the drug delivery systems is of great interest.

Implants are considered to be attractive parenteral drug delivery systems for the long-term application of drugs and of peptides. Poly(lactide-co-glycolide) (PLGA) is the most frequently used and extensively researched polymer for the implant preparation. It is known that the microclimate pH (μpH) in PLGA implants can drop dramatically causing peptide or protein instabilities as well as drug insolubilities and further decomposition. Although the internal pH behavior of PLGA implants and microparticles has been studied in vitro, no direct and continuous investigations about the μpH in in situ forming implants have been published yet. This is caused by the absence of non-invasive methods to measure the pH value in vivo. Thus it is unclear whether in vitro pH measurement results are assignable or not. In this study the μpH of in situ forming PLGA implants were mapped in vitro, in vivo, and ex vivo. A non-invasive in vivo pH measurement method using the multispectral Maestro fluorescence imaging system was explored. Performed in vivo experiments did not only enable to make conclusions regarding the μpH. Also expectations regarding the solvent replacement in the core area of the implant and of the release profile of the hydrophilic substances could be made.
The experiments emphasized the broad application range of the fluorescence imaging technique to non-invasively monitor the μpH values in drug delivery systems in vivo.

**Introduction**

In the last 20 years parenteral drug delivery systems especially for the long-term protein and peptide release have received increasing research interests. They enable controlled release of the incorporated therapeutic agents for time periods lasting over several days, weeks up to months. This enhances the compliance of patient by reducing the application frequency of the therapeutic agents. Furthermore, these depot formulations can minimize undesirable side effects caused by fluctuating drug plasma levels. Already marketed controlled release systems used for the application of peptides are microspheres, solid implants, and in situ forming implants (ISFI) [1; 2]. These systems provide flexible delivery characteristics. However, these possess certain advantages and disadvantages. Microspheres for example have to be thoroughly suspended prior the injection in an oily or aqueous liquid, in order to achieve the complete injection of the entire dose desirable. Solid implants, normally in cylindrical geometry, can directly be deposited subcutaneously (s.c.) via large needles. This however, might be painful for the patient and limits the application doses [1; 2]. Nevertheless, the versatility of possible sizes, different materials and shapes permits the adjustment of drug release rates from solid implants. In situ forming systems can be injected with a syringe through a small needle into the target site, where the implant is formed immediately.

The implants can be formed either by in situ cross-linking of monomers or by in situ solidifying of polymeric materials due to the changes in the environmental pH or temperature. Another method is based on the solvent removal which induces polymer precipitation. Within this approach, the polymeric matrix material is dissolved in a water-miscible, biocompatible solvent. Upon administration, the polymer precipitates due to the replacement of the solvent with the tissue fluids. Potential disadvantages to be considered are the inconsistent shapes of the formed implant, undesired burst effects and possible solvent toxicity [3; 4]. Compared to the pre-shaped parenteral depot systems, biodegradable implants formed by injectable fluids have the advantages being both, less invasive and less painful.
Among plenty of the investigated synthetic and natural polymers the biodegradable PLGA is most widespread utilized for implants. PLGA is well characterized for its safety and biocompatibility record data [1; 5].

PLGA is used in several commercially available parenteral controlled release products like Sandostatin LAR, Lupron Depot, Decapetyl SR, Suprecur MP and Risperdal Consta [6-9]. Two other marked products deploying phase separation by solvent exchange technique based on a PLGA are Atridox [6] and Eligard [7]. PLGA is dissolved in the water miscible, physiological compatible organic solvent N-methyl-2-pyrrolidone (NMP). However, there is controversial data on application of NMP published in the literature. It is described that NMP can cause the degradation of proteins as well as of PLGA and may damage the muscle tissue [10-12]. Emerging scientific data shows that PEG 400 can be an alternative non-toxic solvent for PLGA [4; 13].

Besides the use of the right solvent, another property of the PLGA implants, proved to be critical especially for the delivery of proteins and peptides as well as of poorly water soluble drugs. This is caused by the accumulation of the PLGA degradation products in the implant which are acidic in its nature [14]. In vitro experiments with PLGA microspheres emphasized that the pH value in the microspheres during the incubation period under physiologic conditions can drop from pH 7.4 to approximately pH 3 [15; 16]. This dramatic change in the pH can cause protein instabilities or pH-dependent changes in the drug solubility- as well as decomposition- and deactivation processes [14; 17; 18]. It has been shown, that the pH drop can result in the deiformylation of the incorporated vincristin and in the acid-induced aggregation of bovine serum albumin (BSA) [19; 20]. In addition, also accelerated polymer degradation at low pH-values can cause additional protein instabilities and strongly influence the release profiles. Therefore, the μpH quantification and determination of other affecting factors is critical for the better understanding of PLGA delivery systems and for the formulation design. Thus the methods to determine the microclimate pH (μpH) inside in situ forming implants are of great interest. However, techniques that enable to continuously and non-invasively measure the μpH are missing. Compared to the overall amount of published data based on PLGA drug delivery systems only few articles dealing with the measurement of the μpH. Those are predominantly limited to the in vitro experiments.[16; 19; 21-23]. Many research groups have used indirect methods by measuring pH values in the incubation media with pH-meter and an attached microelectrode [24]. By this, assumptions of the change of the internal μpH and the accumulation of degradation products were
made. These were based on the acidic liberation of degradation products which consequently changed the pH of the incubation media. However, this pH drop of the media may further accelerate ester hydrolysis and thus does not represent the in vivo conditions.

Only a few numbers of techniques are available to measure in vitro the μpH directly in PLGA systems. Potentiometric measurements of μpH values were performed by using the PLGA coated glass electrodes [20]. Within this approach it has been shown that the μpH varied with the thickness of the coating. Highly acidic μpH values (pH 2 - 3.5) were measured after one day of incubation in neutral buffer solution. After one week neutral values (μpH 6.5) were detected in the case of 7 μm thick coatings. Whereas, the μpH remained acidic up to 4 weeks in the case of 30 μm - 250 μm thick films. Others characterized injection-molded implants by puncturing the tissues surrounding the implants with a microelectrode [25]. However, the experimental setup of using an electrode is not suited for small scale systems like microspheres or even for non-invasive in vivo application.

Other groups are dealing with the visualization of acidic μpH values in PLGA microspheres using confocal microscopy with pH sensitive dyes [22; 23; 26]. The confocal laser scanning microscope (CLSM) technique combines non-invasive pH measurements with the visualization of the pH allocation. Therefore, pH sensitive fluorescence dyes like SNARF-1 were incorporated into PLGA spheres. The emission spectrum of this dye undergoes specific pH-dependent wavelength shift. This enables calculating the ratio of the fluorescence intensities based on two emission wavelengths and consequently to determine and to visualize the local μpH value [22]. In vitro experiments with PLGA microspheres using CLSM confirmed a pH drop to pH 3 [15; 16]. Thus CLSM findings proofed potentiometric measured in vitro results and enabled to measure the μpH also in micrometer structures. Nevertheless, light transmission through the measurement object is a prerequisite for CLSM measurements. Therefore, it is challenging to transfer the measurement principles to in vivo studies where they would be by default limited to the skin surface [27].

Other approaches using 31P-Nuclear Magnetic Resonance (NMR) probes [16] or to pH sensitive spin probes which are analyzed by electron paramagnetic resonance (EPR) spectroscopy [21; 28-30]. Until now EPR was the only technique which allowed to demonstrated that pH drops down to pH 2 can occur inside PLGA implants after they were administered s.c. to mice in vivo [28]. However, EPR spectroscopy is limited to the measurement of the average pH values. The visualization of pH distributions in implants by this method is currently not possible.
Although, it can be expected that under in vivo conditions complex factors like perfusion, body liquids, enzymes, elimination processes strongly influence the μpH in PLGA implants the lack of techniques available to measure the μpH in in situ forming implants non-invasively in vivo hampered further research [13; 31; 32]. Former multispectral in vivo fluorescence imaging studies of water soluble polymers and nanocarriers demonstrated, that the technique is able to detect reproducible smallest spectral emission changes of the peak profile in vivo [33-35]. However, information if this technique can also be applied to track in vivo for the first time and in detail μpH changes of in situ forming implants was missing. As in vivo fluorescence measurements of the pH values using non-invasive imaging are hampered by a multitude of influencing factors like auto-fluorescence of skin, lack of capable dyes with sufficient quantum yields, wavelength dependent light absorption, intensity variations, possible bleaching and many others, detailed in vitro and ex vivo experiments had to be performed prior in vivo statements about the pH behavior could be made. Based on the knowledge gained from numerous performed pretests in vivo fluorescence imaging studies were designed in order to non-invasively determine the μpH of the PLGA implants in vivo. In addition comparative pH in vitro EPR studies and in vivo MRI imaging were performed to investigate the implant shape and to compare the obtained findings with previous published results.

**Materials and Methods**

**A 1. Materials**

PLGA (Resomer RG503H, poly(lactide-co–glycolide), molar ratio 50:50, Mw = 34 kDa), was received from Boehringer Ingelheim, Germany. All buffer substances (citric acid/ di-sodium hydrogen phosphate (buffer 1), potassium dihydrogen phosphate/ di-sodium hydrogen phosphate (buffer 2) and mono-sodium phosphate/ di-sodium hydrogen phosphate (buffer 3) as well as 3 Hanna pH standard buffer solutions (pH 4.01, 7.01 and 10.01) were purchased from Sigma Aldrich, Germany. The fluorescence dye SNARF-4F (5-(and-6)-carboxylic acid, pKa 6.4 [36]) was purchased from Invitrogen, Germany. The EPR probes AT (4-Amino-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl, pKa 6.1 [37]) and HM (2,2,3,4,5,5-Hexamethyl-imidazoline-1-oxyl, pKa 4.7 [37]) were obtained from the Institute of Chemical Kinetics and Combustion, Russia. Lutrol 400 (PEG 400) was a kind gift from BASF, Germany.
A 2. Implant preparation

30 % (w/v) of PLGA were dissolved in PEG 400 under stirring at room temperature. Either one of the spin probes AT or HM (400 μg/mL) used for EPR experiments or the fluorescence dye SNARF-4F (40.0 μg/mL) were homogeneously incorporated.

A 3. EPR experiments

The EPR in vitro experiments followed the recently published protocol [38]. Calibration curves of both used pH-sensitive nitroxide spin probes (AT and HM) were made over a pH range from pH 1.0 to pH 12.0 [39]. Therefore, the recorded EPR spectra (1st derivative, measured at 1.3 GHz) were integrated and the distances between the 1st and the 3rd peaks were determined as the magnitude of 2a_n (hyperfine splitting parameter). Corresponding pH-calibration curves were obtained by a sigmoid Boltzman fit. To achieve a sufficient signal-to-noise ratio of the EPR spectra during the in vitro pH measurements 0.2 mmol/L spin probe were added to 50 mL phosphate buffer (buffer 3, 0.1 M, pH 7.4; 37 °C). 200 μL of the prepared implant dispersions (containing the spin probe AT or HM) were injected through a needle (25 gauge) into perforated plastic Eppendorf cups. Immediately afterwards, the cups were placed into 100 mL flasks which contained 50 mL buffer. Thereafter, the flasks were placed on an incubation shaker (30 rpm, 37 °C). For the measurements, the cups were removed from the buffer, dried with a cellulose tissue and wrapped by a plastic foil to prevent drying. Subsequently they were transferred to the EPR spectrometer, measured and afterwards replaced to the buffer after removing the foil. The incubation buffer was exchanged every second day to prevent the accumulation of the degradation products. The EPR spectra were obtained using an L-Band (1.3 GHz) spectrometer from Magnettech, Germany equipped with a re-entrant resonator. The detailed measurement parameters were set as follows: field centre 49 mT, scan range 10 mT, scan time 30 sec, modulation amplitude 0.1 mT. All measurements were performed triplicate, data were reported as mean ± SD (standard deviation).

A 4. Fluorescence imaging

All in vivo experiments complied with the standards for the usage of animal subjects as stated in the guideline from the animal care and use committee of Saxony-Anhalt. The housing, handling and measuring of the nude mice was performed as published recently [33; 35; 40]. 200 μL of the PLGA/PEG 400 dispersion, loaded with
SNARF-4F were slowly s.c. injected into the neck or to both abdominal sides of the thighs, depending on the respective experiment. For the injection as well as prior each measurement mice were anesthetized.

All in vitro and in vivo fluorescence imaging experiments were carried out using the Maestro in vivo fluorescence imaging system from Cambridge Research & Instrumentation (Cri), United States (now PerkinElmer (Caliper Life Sciences), United States and the Maestro software (version 2.10). The equipment was used applying previously investigated measurement settings [33; 34; 40]. Briefly, a Cermax-type 300 Watt Xenon lamp and two, the green and yellow filter-sets were used. Multispectral imaging cube sets were acquired in 2 nm steps using automatic exposure times. Both filter sets were used simultaneously for the generation of each measurement file. Respective emission spectra as well as an auto-fluorescence signal as background were defined based on reference in vitro and in situ pH measurements. Therefore, Eppendorf cups with buffer solutions of defined pH values were placed s.c. under the skin of three sacrificed mice. The generated reference library allowed the visualization of the pH distribution as well as allocation of the dye. In addition averaged emission spectra of different regions of interest (ROI) were extracted from the measurement cubes. The ratios of the emission maxima were determined and corresponding μpH values were calculated.

### A 5. Benchtop Magnetic Resonance Imaging (Bt-MRI)

Bt-MRI measurements were performed using a 20 MHz NMR benchtop system (prototype based on Maran DRX2, Oxford Instruments Molecular Biotools, UK). The system was equipped with a 23 mm sample access. The mice were placed on glass slides with a fixed inhalation mask for anesthetization. Same measurement parameters were used as published earlier [13]. Briefly, a standard spin-echo sequence was used with a spin echo time (TE) of 9.8 ms and a repetition time (TR) of 178 ms. 16 averages were applied resulting in an acquisition time of about 352 s for each image. The field of view was 40 mm x 40 mm had a resolution of 128 x 128 points.
Results and Discussion

A 6. In vitro EPR pH measurements

Non-destructive EPR spectroscopy was chosen as an established method to measure μpH values in vitro [29; 41]. The EPR experiments were performed to provide in vitro information as basis for future in vivo fluorescence imaging experiments. To achieve sufficient signal-to-noise ratio even over the long time release process, the pH sensitive spin probe was incorporated in both, the polymeric solutions and the incubation buffer. To assure that the spin probe containing buffer does not interfere with the μpH determination in the implants, the absorbed buffer was carefully removed prior the EPR measurements.

The used nitroxide probe is sensitive to changes of the μpH [41] as well as to the micro-polarity and micro-viscosity. Therefore, the spin probes migrated with the solvent outside the implant as the incorporated hydrophilic spin probes were readily soluble in PEG 400 and did not precipitate within the hydrophobic polymer matrix. After 6 h no solid signals were no more detectable indicating that the solvent exchange has been completed. This is in accordance with the previous results [13].

Due to the fact that the solvent replacement dominated in the first day of incubation at all investigated implants, no reliable data about the μpH within the first 24 hours could be obtained. Calculated pH values based on performed in vitro measurements are shown in Figure 1. It can be concluded that the pH decreased of pH within the first 6 days below pH 3 is caused by the accumulation of acidic degradation products in the implants.

![Figure 1: Time dependent pH change of PLGA implants, determined in vitro by EPR spectroscopy.](image-url)
Afterwards, due to the increased pore formation, accompanied by medium exchange, the pH inside the implants rose again to the neutral range after 21 days. Under the current conditions the EPR method was restricted to in vitro measurements. Therefore, further in vitro and in vivo experiments were focused on fluorescence imaging experiments investigating if the observed pH drop within the first week occurs also in vivo.

A 7. Evaluation a pH measurement method using fluorescence imaging

Many different fluorescence imaging systems are currently on the market [42]. Compared to those the Maestro imaging system allows multispectral analysis [43-45] and the export of spectral data as an ASCII-file, the American Standard Code for Information Interchange. Based on the Maestro system, emission spectra can be extracted from each pixel of the measurement cube but also from a group of pixels in a desired region of interest (ROI) within the cube [30]. This allowed both the external analysis of spectral intensities in spreadsheets as well as the image processing using the Maestro auto-fluorescence removal tools. To use these functionalities for in vivo pH measurements of PLGA implants, extensive preliminary in vitro experiments had to be performed. Therefore, different possibilities of pH calculations based on the spectral intensity values were evaluated. Due to the fact, that fluorescence intensities may vary in in vivo surroundings, dyes with pH-dependent wave length shifts of the emission spectra should be used [16; 22; 46]. Another important prerequisite for in vivo measurements is the necessity of using dyes with emissions above 550 nm, otherwise the emission of the dye would be overlaid by the auto-fluorescence of the skin. The SNARF dyes were the only commercially available ones which fulfilled these both requirements. Within this group of dye, SNARF-4F has the lowest pKa value (pKa of about 6.4). Dyes with low pKa values are needed to measure acidification processes in implants. Thus SNARF-4F was chosen to calculate pH values within this study. The chemical structure and the pH dependent equilibrium of SNARF-4F are shown in Figure 2. The chemical structures of the SNARF dyes exist like fluorescein in a ‘closed’ lactone and an ‘open’ quinoid form. The lactone form is dominant especially in non-aqueous environments and is non or only very weak fluorescent known also from fluorescein [47-49]. The ring opening of the lactone and its subsequent ionization at the different acidities (anion) of the microenvironment results in characteristic fluorescence emission maximums at two different wavelengths (using the Maestro system at 606 nm and 648 nm, see also Figure 3).
The pH dependent equilibrium can be used to measure fluorescence ratios reliably and to quantitatively determine pH values [48].

![Figure 2: Chemical structure and the pH dependent equilibrium of SNARF-4F](image)

The disturbing auto-fluorescence for s.c. measurements is mainly caused by the skin. To reduce the influence of spectral changes of the emission light due to the skin passage several measurement and calculation approaches were tested. The auto-fluorescence occurs mainly below 650 nm. Thus using two filter sets within one measurement, the green and the yellow one emphasized to be best suitable. Two filter sets reduced the influence of the auto-fluorescence signals. The obtained pH dependent emission spectra of SNARF-4F are shown in Figure 3.

![Figure 3: Normalized emission intensities of SNARF-4F in two different buffer solutions: pH 5 (green) and pH 7 (red), measured with the green filter set (line) and the yellow one (dashed). Three vertical, black lines refer to the emissions at 606, 648 and 668 nm, used for pH calculations.](image)
Subtracting the intensity value at 668 nm (yellow filter set) from that measured at 606 nm (green filter set) and dividing the result by the intensity value at 648 nm (green filter set) was evaluated to be the best method for further pH calculation. The subtraction step reduced the influence of spectra broadening due to the emission spectra disturbing auto-fluorescence signals. The dividing step in the calculation eliminated the influence intensity variations due to both, varying dye concentrations as well as of different exposure times. The calculated pH dependent ratios are displayed in Figure 4 a. Reproducible pH values were also detected when measurements were performed with exposure times below or above the optimum.

![Graphs showing pH calculations](image)

**Figure 4:** (a) Calculated ratios of pure pH buffer solutions as well as after varying dye concentration and exposure time. (b) Influence of PLGA/PEG400 to the pH calculation. (c) Boltzmann plots and respective raw data of *in vitro* and s.c. *ex vivo* measured pH solutions. (d) Boltzmann plot (blue) and limits of reproducible (black lines, at pH 5 and 7) and sufficient (red lines) pH calculation.
The same method has been used to measure the pH values of buffer solutions after PLGA / PEG 400 mixtures where added. The results are displayed in Figure 4 b. The experiment results emphasized that the addition of the implant polymer has no influence to the reliable detection of the pH values.

The spectral shape of the detected emission spectra can be changed if the emitted light is passing tissues like the skin. This could falsify calculated pH values. In order to investigate this influence buffer containing Eppendorf cups were measured subcutaneously served for the calculation of a reference Boltzmann plot. Three dead mice varying in age (3, 6 and 12 months) and consequently in skin thickness and partly in skin compositions were used to increase the robustness of the resultant ratios. Obtained original measurement images of the two Eppendorf cups (pH 5 and pH 7) are shown in Figure 5. The resulted Boltzmann plot in comparison to the in vitro measured one is displayed in Figure 4 c. A shift of the ex vivo measured ratios to lower values has been observed.

The ex vivo measured Boltzmann plot was used for all further calculations. As depicted in Figure 4 d pH values can be calculated based on the obtained emission intensity ratios within the pH range of pH 4.5 to pH 7.5 (marked by red lines). However, determinations below pH 5 and above pH 7 are highly error-prone as small variations in calculated ratios highly influence the calculated pH value. Thus, fluorescence pH measurements can only be reproducibly performed at the range between pH 5 and pH 7 (marked in Figure 4 d by black lines).

It is also evident in Figure 4 d that error bars of calculated ratios increase to higher pH values. This is can be explained by the emission spectra drop down at 606 nm (cp. Figure 3). Thus, the intensity value used for the pH calculation is highly influenced by the auto-fluorescence of the skin.

Further ex vivo experiments followed in order to investigate the optimum dye concentration. Therefore, Eppendorf cups with the buffer solutions varying in pH (pH 5, pH 6 and pH 7) and in dye concentrations (0.25, 0.50, 0.75 and 1.00 μg/mL)
were analyzed. The results are shown in Figure 6 a. It has been observed that low pH values can be reproducibly measured even at low dye concentrations. Same results were found at pH 6 although the results were less accurate. For buffer solutions of pH 7 a trend to lower pH values was identified if the dye concentration is reduced. Due to the decreased emission intensities the influence of the auto-fluorescence of the skin increased. As discussed above, the background signal influences especially the intensity value at 606 nm (cp. Figure 3). Consequently this has an impact to the calculation of higher pH values (pH 7) if samples are low concentrated. Based on the experimental results it can be concluded that SNARF-4F concentrations of 1.00 μg/mL or above are optimal for reproducible pH calculations.

As dye concentrations can not be determined in vivo, cumulative signal to noise ratios were calculated and evaluate in order to ensure correct pH determinations. Therefore, in vitro data shown in Figure 6 a was used to calculate cumulative signal to noise ratios for all tested concentrations. As it is visible in Figure 6 b, reproducible pH values can be calculated if the calculated cumulative signal to noise ratio is 20 or above.

Additionally to the pH calculation method also the visualization of the pH allocation using the Maestro imaging system was evaluated in vitro. Therefore, emission reference spectra of pH 5 and pH 7 (cp. Figure 3) were defined for the spectral library. Thus, each pixel of the multispectral measurement cube could be assigned in the automatic unmixing process by the software to the defined emission reference spectra of pH 5 or pH 7 considering also ratios between both of them. The pixel is
displayed in the color which was manually pre-assigned to the respective reference spectrum. This allowed visualizing the pH allocation in the desired measurement range of the SNARF-4F dye. If no conformity was found the pixel has been displayed in black.

The reproducibility of the image processing was evaluated with the PLGA / PEG 400 dispersion planned to be used in the upcoming in vivo experiments. The mixture was placed on a flat bowl and imaged time dependently before and after adding buffer solutions varying in pH. Figure 7 a shows the obtained and unmixed RGB images. Pixels which were allocated to pH 5 are displayed green; others, which were assigned to pH 7 are red. The background signal was set to black.

As it is seen in Figure 7 a (A) the dye is quenched nearly completely in pure PLGA/ PEG 400 solutions. Only slight fluorescence signal was detectable. It can be expected that the absence of water promotes the formation of the lactone form which is non-fluorescent. After adding buffer solution with pH 7 PEG 400 mixed with water, the entrapped dye immediately got fluorescent as seen in Figure 7 a (B). Even smallest amounts of water result in a ring opening of the lactone. The fluorescence intensity increased within the continuous permeation of water (Figure 7 a (C)). As PLGA is not water-soluble it precipitated immediately and the implant formed.
As shown in Figure 7 a (B and C) after a few seconds all pixels of the fluorescent PLGA implant in the obtained image were allocated by the Maestro software to the reference spectra of pH 7 and thus displayed in red. Afterwards, the pH of the surrounded buffer solution was adjusted to pH 5. Consequently also the buffer in the surface of the PLGA implant decreased. As it is visible in the images of Figure 7 a (C to E), the green amounts in the images increased over time. This evidenced by the observed pH change from pH 7 to pH 5. After 3.3 min nearly all pixels of the PLGA implant were allocated to the emission spectra of pH 5 (Figure 7 a (F)). The experiment verified that the visualization of pH values within the pH rage between pH 5 and pH 7 is possible.

The underlying data of the RGB images shown respectively in Figure 7 a were used to calculate additionally the total signals for pH 5 as well as for pH 7. Therefore, the intensity ratio between pH 5 and pH 7 of each pixel of all single measurement files was calculated. All intensity values of the pixels assigned pH 5 or to pH 7 were summated to the respective total signal. The resulted values are displayed in Figure 7 b and confirmed the visual findings. Nearly no fluorescence has been detected at the beginning of the experiment. After adding the buffer solution (pH 7, marked by the first black line, A), the total intensity increased rapidly. After 45 s the buffer pH was lowered to pH 5 (marked by the second black line, B). Consequently, the total intensity of the detected pH 7 signal decreased continuously and reached zero after about 2 min. During this time the total intensity of pH 5 increased. The maximum total signal was measured after approximately 90 s. Thereafter, the total signal of the hydrophilic SNARF-4F dye decreased continuously. This can be explained by the release of the dye out of the implant, by what the total pixel intensity decreased.

A 8. In vivo dye distribution studies

First, in vivo studies were initiated by injecting the PLGA / PEG 400 dispersion containing the SNARF-4F dye into the abdominal side of the both thighs. The mixture itself was visually colorless. Time-dependent images after 15 min, 60 min and 48 h are shown in Figure 8. The fluorescence signal assigned to the dye emission spectra was colored in magenta. 15 min after injection a distinct fluorescence signal was detectable. This is caused by the exchange of PEG 400 with the aqueous body fluids. The measurable fluorescence intensity increased as it was also observed in the in vitro experiment. The maximum intensity was measured after about 60 min indicating that most of the PEG 400 was already exchanged. This is in accordance with the previously published in vitro EPR results [13]. Furthermore, as seen in
Figure 8, the physically entrapped dye is spread from the injection site into the surrounded body tissue. The solidified PLGA implants at both sides of the thighs are still detectable after 2 days indicating that sufficient dye amounts were still entrapped in the PLGA implants. Released dye molecules diffused to the surrounding tissues and were eliminated quickly as the dye is hydrophilic.

![15 min](image1) ![60 min](image2) ![48 h](image3)

Figure 8: Time dependent, unmixed RGB images of the abdominal site of a mouse. The isolated SNARF-4F signal was colored in magenta. The PLGA / PEG mixture was ventrally s.c. injected to both sites of the hind legs.

The implant was deformed over time due to the movement of the mouse between the experimental measurements. This hampered surface analyzes. Therefore, the injection site was changed for followed long-term experiments. The PLGA / PEG 400 mixture was henceforth injected into the neck of the mice subcutaneously. The resultant non-invasively measured images of a mouse using the 'compared' jet color visualization method are displayed in Figure 9. This function enabled to display SNARF-4F emission intensity distributions independently from the pH. In addition, it facilitated to compare images of different measurements even if they were captured with different measurement settings like exposure times and binging. Thus, time dependent image analysis of intensity changes can be performed.

Already 3 hours after injection, wide parts of the already formed implant were displayed in dark red indicating high fluorescence intensities. This confirmed previous in vivo experiment (see Figure 8) and is in concordance with previous ESR and MRI studies [13]. These former studies showed that initially a thin polymer shell was formed that entrapped the PLGA / PEG 400 solution. Within the further exchange of PEG 400 by water through the thin shell the polymer precipitation process proceeded. The solvent / non-solvent exchange observed was very fast, after 1 h about 70 % of the PEG 400 was replaced by water [13]. This has been also confirmed by the present study. The outer edge of the implant is indistinct and light blue confirming the diffusion of the dye out of the implant. Still 1 day after injection wide parts of the implant were highly fluorescent. Therefore, it can be concluded that small amounts of the aqueous body liquids were still present in the matrix. The
fluorescence intensity decreased however within the next 3 days. This can be explained by the discharge of remaining hydrophilic body liquids out of the implant and the final solidification of the implant matrix. Thus, the remaining dye in the implant is quenched. 4 days after injection the intensity at was lowest level indicating that the implant was completely solidified. As PLGA is insoluble in aqueous solvents liquids are removed during the solidification and equilibrium of the dye is shifted to the non-fluorescent form.

Figure 9: Time dependent, intensity weighted jet color images of the SNARF-4F signal of a PLGA / PEG 400 implant, injected to the neck of a mouse.

The fluorescence intensity increased continuously between day 6 and day 20. This can be explained by the accumulation of acidic PLGA degradation products [14] which accumulated in the implant [31]. During this process, more and more pores are formed and small amounts of water can further pass into the implant. Comparable results were also obtained from the pre-shaped PLGA implants [50]. It is furthermore detectable, that the size of the implant remains nearly constant until day 20 and decreased during the next 10 days gradually. This size reduction can be explained by the progressive softening of the implant. Through the formed pores the degradation products as well as part of the entrapped dye can diffuse out of the implant. The dye was eliminated nearly completely 30 days after injection.
A 9. *In vivo* and *ex vivo* investigation of implant size and implant pH allocation

Fluorescence imaging as well as MRI were also used to investigate the size and the position of the implant in the body. Benchtop MRI (BT-MRI) was applied as a complimentary *in vivo* method to non-invasively visualize the shape of the implant. The used BT-MRI is an alternative low cost system based on permanent magnets [51; 52]. It is successfully utilized for the characterization of tumors and of PLGA implants [13; 53]. It has been shown, that the contrast and the signal intensities between the PLGA / PEG 400 implant and the surrounding tissue were sufficient to distinguish both substances, even without adding additional contrast agents [13]. Due to high PEG 400 amounts at the beginning, the injected polymer solution had nearly the same relaxation times as s.c. fat and appears bright [13]. As it has been observed in the fluorescence images the PEG 400 was quickly exchanged with water, causing PLGA solidification. The solidified PLGA implant was imaged by MRI 9 days after injection (Figure 10 a). The implant (marked by arrow) appeared black. Thus it can be easily distinguished between the brighter skin, the fat tissues and the implant. The shape of the implant can be described as a thick disk. This is the result of the compressive tissue forces *in vivo* [54].

After the BT-MRI measurement, the implant was excised, sliced in the middle and analyzed by fluorescence imaging technique. RGB images of the implant cutting section are shown in Figure 10 b and c. In Figure 10 b different colors were assigned to the emission reference spectra of pH 4.5, pH 5.0 and pH 5.5. Within the unmixing step the Maestro software tags all pixels with an emission spectrum related to the reference of pH 4.5 in blue. Pixels which were assigned to pH 5.0 were displayed yellow and those which were allocated to pH 5.5 red respectively. In between a slight graduation of the respective mixed colors occurred. This enabled visualisations of pH distributions in the implant. As it can be observed from Figure 10 b, areas with lowest pH values were found in the middle of the implant. Higher acidity in the center was caused by the accumulation of the degradation products and is in accordance with the formerly published *in vitro* results of PLGA microspheres [31]. Regions with the higher pH values were primarily detected in surface areas of the implant especially near the skin. An increased pH under the skin can be explained by the better s.c. perfusion by what acidic degradation components are eliminated faster.
Figure 10: (a) *In vivo* MRI lateral profile picture of a mouse, 9 days after injection. The solid PLGA implant is visible. (b) *Ex vivo* fluorescence RGB image of the same, excised implant. The blue color is related to areas with the lowest detected pH values (pH 4.5), green and yellow indicate an increased pH and red regions are assigned to pH 5.5. (c) Intensity weighted jet color image of the same implant indicating areas with highest dye concentrations.

The intensity allocation in the implant is shown in Figure 10 c. Highest fluorescence intensities were measured in the middle of the implant. This is in concordance with the previously discussed results and is caused by the two factors. First, dye molecules in outer regions diffuse out of the implant during the solidification, directly after the injection. Second, the dye is only weak fluorescent in pure PLGA surroundings.

Due to the light properties, excitation and emission, light in the visible bandwidth has limited penetration depth. Thus emitted light of excited SNARF-4F dye molecules can only pass a few millimeters depending on the dye concentration. By this, an *in vivo* measured pH represents the surface pH values. In the case of the implant shown in Figure 10 b only areas which were assigned to pH values around pH 5.5 and displayed in red and yellow would be non-invasively detectable *in vivo*.

**A 10. In vivo pH measurements based on fluorescence imaging**

Based on the previous *in vitro* and first *in vivo* results, long-term *in vivo* study with nude mice was conducted to measure non-invasively the pH of PLGA implants. The analyzed results of three mice are shown in Figure 11 a.

The presented pH value is the average pH of the total surface area of the whole implant. The red line defines the upper pH detection limit (pH 7.0) and the green one, the lower pH calculation limit at pH 5.0. In between this bandwidth, pH values could be measured *in vivo* for more than 25 days. The pH remained constant for the first 2 -
3 days which conforms the above discussed in vivo dye intensity analysis. After 3 days, the pH decreased continuously due to the accumulation of acidic degradation products down to the lower pH detection limit of pH 5.0 [13; 31].

Figure 11: (a) Time dependent in vivo pH values of PLGA implants, injected into the neck of 3 mice. The 3 black dots represent pH values of an excised, sliced implant. The 2 horizontal lines mark the upper and the lower pH detection limit. (b) Cumulative signal to noise ratios of in vivo fluorescence imaging measurements. Black line marks the lower limit of reproducible pH determination.

The pH values of all three implants correlated well. This acidification catalyzes the further polymer degradation process. Generally, these results are in accordance with the performed in vitro EPR experiment. However, the process of the pH decrease was found to be slower in vivo. In Figure 11 a it is depicted a reversal kick point after which pH increases for mouse 1 at day 17 and for mouse 3 after day 23. This time point depends on the size and the geometry of the implant. Due to the strength of the skin and the mouse muscles there is an outer force to the implant. This influences the implant surface to bulk fluid volume ratios which have a strong impact on the degradation behavior [11; 32]. Degradation products are accumulated in the center of the implant. Over time period, the small pores are formed. Both effects cause deformation and corruption of the implant as soon as the center is highly destabilized. Consequently, acidic products are exchanged with body liquids which results in an increase of the pH value in the implant. For mouse 1 (Figure 11 a), the pH increase began at day 17. 27 days after the injection the implant was destabilized completely and the dye diffused out of the implant. Thus the fluorescence dye concentration decreased and the cumulative signal to noise ratio fell below the previously defined limit (Figure 11 b). After day 27 the implant of mouse 1 was excised and sliced through the middle. Ex vivo pH values from the top, the upper middle and from the center of the cutting area were measured. As it can be seen in
Figure 11 a (black points) the spectrum of the pH values in the cutting area is quite high. While, the pH at the surface was measured to be 6.3, the pH in the middle was still 5.0. The pH of 5.75 determined in the upper half which is in accordance to the in vivo measured pH values. Based on the final height of the excised implant of approximately 4 mm the measurement depth of the fluorescence imaging method can be expected to be approximately 1.5 mm.

![Figure 11: pH values distribution](image)

In order to investigate the pH distribution in more detail, additional analysis was performed for one implant where the pH was determined in vivo on an intended line from one side of the implant via the middle to the other side of the implant. The results are shown in Figure 12. Within the first 2 days lower pH values were calculated for the outer regions of the implant. This can be explained by the decreased dye concentration in those areas. Thus the cumulative signal to noise ratio falls below the defined detection limit. As it has been previously discussed for Figure 11 b this leads to an error in the pH calculation for pH values between pH 6 and pH 7. Thus lower values than the actual ones are calculated. The pH decreased within the first 4 to 6 days at nearly constant speed. After 9 days, lowest pH values were detected in the center of the implant confirming the above discussed results.

**Conclusion**

PLGA solutions in organic solvents have been extensively investigated as potential novel drug depots over the last decade. However, the uncontrolled pH drop in PLGA systems is one of the most critical factors for the instability of the encapsulated drugs.
or proteins in PLGA controlled release drug-delivery-systems. Therefore, it is important to measure the microenvironment in PLGA systems and to understand the relationship between pH shift and the implant degradation. Though, systemic investigations regarding the internal pH of the formed implants were missing. So far there exist no analytical methods to determine and visualize directly the in vivo changes in the microclimate pH (μpH) in a continuous and nondestructive manner within the same sample on the same animal model. Due to their non-destructive nature, EPR spectroscopy and fluorescence imaging were chosen for serial μpH measurements of the PLGA based in situ forming depots. In vitro EPR experiments showed that during incubation under ‘physiologic’ conditions the pH-value in the system can drop from pH 7 to values around pH 3. Extensive in vitro and ex vivo fluorescence imaging experiments were performed to evaluate the influence of factors like auto fluorescence of skin, dye selection, light absorption, intensity variations and measurement conditions. Based on the obtained information an in vivo fluorescence imaging method was evaluated. It has been demonstrated within this study that the μpH of PLGA implants could be non-invasively measured in complex in vivo surroundings. Due to the lack of the alternative dyes the lower detection limit for the in vivo fluorescence μpH measurements is considered to be pH 5. The physical entrapping of the hydrophilic dye enabled the measurement of the pH as well as of the dye release profiles from the implant. The results are in concordance with the performed in vitro EPR as well as in vitro confocal laser scanning microscopy results. However, the pH drop caused by the PLGA degradation products appears to be slower in its nature in in vivo conditions. This could be explained by different interactions with body liquids and due to varying implant geometries in vivo. The current study demonstrated that the evaluated fluorescence imaging method proves to be an efficient method to non-invasively measure μpH values in vivo.

**Literature**


Supplemental material

(c) Characterization of in situ forming implants for potential controlled API release


Supplemental material

(c) Characterization of in situ forming implants for potential controlled API release


Supplemental material
(d) Investigating alternative application fields of fluorescence imaging


Monitoring of internal pH gradients within multi-layer tablets by optical methods and EPR imaging

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A B S T R A C T

The high variability of gastrointestinal pH is a general challenge regarding constant release from oral drug delivery systems, especially for ionisable drugs. These drugs often show a pH-dependent solubility and therewith associated intra- and inter-individual variability of emerging drug plasma levels. Several strategies have been investigated with the intention to influence the microenvironmental pH (pH(μ)) within solid formulations and therefore achieve pH-independent release profiles. Because of the heterogeneity of solid systems, a precise prediction of the occurring pH(μ) is rather difficult. It is therefore important to monitor the pH(μ) within the formulations to achieve requested release as well as to minimise pH-dependent degradation processes of the active compound. The purpose of the current study was the analysis of pH(μ) gradients within 2- and 3-layer tablets during hydration using 3 different techniques for comparison intensions, in particular a pH indicator dye, fluorescence imaging and EPR imaging. The influence of the presence or absence of pH modifying substances and of an additional lipophilic inter layer on the pH(μ) was investigated as well as the variation of matrix forming excipient and buffer pH. The influence of the pH(μ) on drug release was analysed as well. In addition, benchtop MRI was accomplished to gain a deeper insight on the hydration and erosion behaviour of 2- and 3-layer tablets.© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The oral route is still the most commonly used way for the application of drugs because of its convenient administration leading to high patient compliance. However, the variability of physiological conditions within the human gastrointestinal tract (pH, gastric residence time, intestinal motility, food intake) can be a serious challenge for a predictable release and effect of oral drug delivery systems (Grundy and Foster, 1996). Especially the variability of the gastrointestinal pH has shown to be an important parameter for drugs with ionisable functional groups (weak acids/bases). In most cases, the unionized form shows a low aqueous solubility leading to changed solubility under acidic (stomach) and neutral (intestine) conditions. The dissolution rate of a drug with diffusion-controlled release behaviour is dependent on the solubility of the drug in the diffusion layer (Gibaldi, 1984). Thus, pH-dependent solubility may lead to incomplete drug release and remarkable intra- and inter-individual variability of emerging drug plasma levels.

The concept of microenvironmental pH (pH(μ)) is often used in conjunction with solid formulations characterising the pH, which is generated within the formulation during hydration by surrounding media or humidity (Siepe et al., 2006; Badawy and Hussain, 2007). The pH(μ) has shown to affect drug stability inside solid formulations as well as dissolution behaviour, both influencing the bioavailability of an active compound (Badawy and Hussain, 2007). For this reason, several attempts have been published with the intention to modify and measure the pH(μ) within solid formulations to achieve pH-independent release or enhance storage stability of weakly acidic and basic drugs. One strategy is the incorporation of enteric polymers into hydrogel matrix devices. These polymers show a pH-dependent solubility and are supposed to act as pore formers (Akiyama et al., 1994; Streubel et al., 2000) and pH modulators (Tatavarti et al., 2004) for weakly basic drugs. Another attempt is to influence the pH(μ) by incorporation of pH modifying substances. Organic acids, showing different solubilities and acid strengths, were used to enhance the release of weakly basic drugs (Thoma and Zimmer, 1990; Streubel et al., 2000; Varma et al., 2005; Siepe et al., 2006; Tatavarti and Hoag, 2006; Gutsche et al., 2008). On the other hand, basic salts were reported to improve the release of weak acids (Doherty and York, 1989; Riis et al., 2007; Tran et al., 2008). The pH(μ) is influenced by many factors including excipients, active compounds, amount of water penetration, diffusion processes and pH of surrounding media. Therefore, a certain prediction is rather difficult. There is a need to monitor the local pH within solid formulations to optimise the pH(μ) regarding drug stability.
and requested drug release. Although the pH of solutions is easy to determine potentiometrically, it is much more challenging to analyse the pHs of solid or nearly solid formulations. Several techniques were used to gain information on the pHs; however, there are no well-established methods available for all purposes. Diffuse reflectance spectroscopy was used to determine the pHs of dry tablets (Gombitza et al., 1994; Gombitza and Schmidt, 1995; Scheef et al., 1998; Zschuck et al., 2005; Pudipeddi et al., 2008). However, only the surface pH could be determined and possible interactions between the pH sensitive dye and excipients should be kept in mind. Incorporation of pH indicator dyes and following examination of occurring colours over time of hydration was also reported (Streubel et al., 2000; Varma et al., 2005; Adhikary and Vavia, 2008; Ching et al., 2008). This dye method was easy to apply but only a rough, imprecise estimation could be obtained. To achieve information concerning pHs within the tablet core during contact with buffer, tablets had to be cross-sectioned. Another attempt was the usage of a surface pH electrode to analyse the surface pHs of solid dispersions (Tran et al., 2008) as well as the pHs of cryosections of hydrated tablets (Gursche et al., 2008). Again, to gain insight on the pHs of the inner regions, tablets had to be cut in pieces. Confocal laser scanning microscopy was used to non-invasively image pH sensitive fluorescent dyes, giving a spatial resolution of pHs (Cope et al., 2002; Li and Schwendeman, 2005). One restriction of this technique is the limited object size, thus, only eroding microphases were analysed. Electron paramagnetic resonance (EPR, ESR) spectroscopy allows the non-invasive detection of paramagnetic compounds. The majority of drug delivery devices are not directly detectable by EPR because of the absence of naturally occurring radicals. Thus, it is necessary to incorporate paramagnetic substances e.g. stable nitroxide radicals with the objects of interest. Dependent on the used substance (so called spin probe), information about microviscosity, micropolarity and pHs inside drug delivery systems can be obtained based on the spectral sensitivity of the nitroxides to their environment (Mader et al., 1997; Brunner et al., 1999; Lurie and Mader, 2005; Kempe et al., 2010). EPR imaging now combines spectral information with the spatial distribution of a spin probe. Therefore, EPR imaging can be used as continuous, non-invasive technique for the spatial determination of pHs within hydrated devices.

The purpose of the current study was the analysis of pHs gradients within multi-layer tablets (2- and 3-layer tablets). Multi-layer tablets can be used for different purposes. It is possible to separate incompatible substances as well as to combine immediate- and prolonged-release profiles of an active compound. Furthermore, floating multi-layer tablets were developed for gastric retention consisting of a floating and a drug-containing tablet layer (Ingani et al., 1987; Wei et al., 2001; Rahman et al., 2006). The aim was to investigate the influence of (1) the presence or absence of pH modifying substances within tablet layers, (2) the variation of matrix forming excipients, (3) the variation of the pH of surrounding buffer and (4) the incorporation of an additional lipophilic inter layer on the pHs within multi-layer tablets. The influence of the pHs on the drug release of two model drugs, Metformin-HCl and Ketoprofen, was also analysed. An internal buffer system (IBS) composed of citric acid and diiodium hydrogenophosphate was used as pH modifier. The IBS was incorporated either in one or two tablet layers to generate a pHs gradient within the tablets. Furthermore, different matrix forming excipients were analysed for their ability to maintain a specified pHs over time of buffer contact. Hydroxypropylmethyl-cellulose (HPMC) was analysed as most frequently used hydrogel polymer which is able to form hydrogel matrices upon contact with water. HPMC is a non-ionc cellulose ether forming a stable hydrogel over the pH range of 3–11. Kollidon SR, a commercially available excipient in the form of a physical mixture of 8 parts of polyvinylacetalate (PVAc) and 2 parts of polyvinilpyrrolidone (PVP), was used as well. Kollidon SR shows excellent tabletting properties and can be used for direct compression (BASF AG, 1999). Because of its aqueous solubility, PVP acts as pore former during contact with water and therefore facilitates drug diffusion. Sponge-like matrices can be observed after 12 h of buffer contact. Three different techniques were used to determine the pHs within multi-layer tablets for comparison of results regarding application spectrum and expenses, in particular, a pH indicator dye, fluorescence imaging and EPR imaging. In addition to the analysis of the pHs, the hydration behaviour of 2- and 3-layer tablets was monitored using nuclear magnetic resonance imaging (NMR-imaging/MRI) in order to gain a deeper insight on proceeding hydration and erosion processes during contact with buffer. MRI has proven to be a non-invasive, well established method to investigate drug delivery systems in vitro and in vivo (Richardson et al., 2005; Metz and Mader, 2008; Nott, 2010). A commercial, low-cost benchtop MRI (BT-MRI) system was used as alternative to common superconducting MRI machines. Recently, BT-MRI has been successfully used to characterise different solid drug delivery devices (Metz et al., 2007; Strübinger et al., 2008a,b; Malaterre et al., 2005). Therefore, BT-MRI was intended to provide detailed information about the differences in the hydration behaviour of 2- and 3-layer tablets.

2. Materials and methods

2.1. Materials

Kollidon® SR was kindly supplied by BASF, Ludwigshafen, Germany. Disodium hydrogenphosphate dihydrate and citric acid were obtained from Carl Roth GmbH & Co KG, Karlsruhe, Germany. They were ground in a mortar and passed through a 250 µm sieve for further use. Aerosil® was purchased from Evonik Degussa GmbH, Essen, Germany. Magnesium stearate was obtained from Magnesia GmbH, Lüneburg, Germany. Lactose-monohydrate was purchased from Euro OTC Pharma GmbH, Röthen, Germany. Methocel K100 CR was kindly supplied by Colorcon GmbH, Idstein, Germany. The fluorescence dye Carboxy SNARF®-1 was purchased from Invitrogen GmbH, Darmstadt, Germany. Bromocresol purple was obtained from Merck KGaA, Darmstadt, Germany. EPR spin probe 4-Amino-2,2,5,5-tetra-methyl-3-imidazoline-1-oxyl (AT) was obtained from N.N. Vorozhtsov Institute of Organic Chemistry, Novosibirsk, Russia. Metformin-HCl was purchased from Biotrend Chemicals AG, Wangen/Zurich, Switzerland. Ketoprofen was obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany.

2.2. Preparation of tablets

The powder mixtures for the manufacturing of tablets were prepared according to compositions shown in Table 1 by blending all ingredients except magnesium stearate with pestle and mortar for 10 min. After adding magnesium stearate, the mixtures were blended for another 2 min. For the preparation of 2- and 3-layer tablets, weighed amounts of the different layers were fed successively into the die of the tablet press and precompressed manually. The final compression force was adjusted to receive tablets with a crushing force of 75 N after compression. Biconvex 2-layer tablets consisting of 200 mg of KSR-P or KSR layer and 100 mg of HPMC-P or HPMC layer were prepared by direct compression using a rotary tablet press (RL 12, Kilian GmbH & Co KG, Germany). Resulting 2-layer tablets had a weight of 300 mg and a diameter of 9 mm. Furthermore 3-layer tablets with an additional inter layer of 50 mg of glycerol monostearate were produced. The inter layer should achieve a better adhesiveness of both layers and decrease diffusion processes between the layers. All analysed tablet preparations are illustrated in Fig. 1.
2.3. Micro acidity measurements using a pH indicator dye

Tablets, containing bromocresol purple (1 mg/tablet) as pH indicator dye, were prepared as described before. These tablets were subjected to 100 mL of a citric acid/phosphate buffer consisting of 0.01 M citric acid solution and 0.02 M disodium hydrogenphosphate solution in a ratio of 4:1 with a resulting pH of 3 (pH 3). The pH 3 of the surrounding buffer was used as typical pH of the late phase of the fed stomach (Jantarad et al., 2008) which is important especially for gastroretentive systems. Photographs of the tablets as whole and cross-sectioned were taken after predefined time intervals (10 min, 2 h, 4 h and 6 h) of contact with buffer with a digital camera (µ850 SW, Olympus, Japan). Every tablet could be analysed only once, therefore, a new tablet incubated in the buffer for the dedicated time interval was used for every photograph. The pH of the buffer was analysed regularly and showed a stable pH of 3.

2.4. Micro acidity measurements using multispectral fluorescence imaging

2-layer tablets, containing the fluorescence dye Carboxy SNARF-1 (0.2 µmol/g powder), were used. The tablets were placed into tubes with the diameter of the tablets and two open ends to allow a constant measuring area and a one-dimensional hydration only from top and bottom of the tablet. The tubes with incorporated tablets were transferred to 100 mL of buffer pH 3 (see Section 2.3). They were removed from the buffer at different time points and analysed by fluorescence imaging. The measurements were done with a Maestro™ in vivo imaging system (Cambridge Research & Instrumentation, Woburn, USA). A green and a yellow filter set were used. Multispectral imaging cube sets were acquired in 2 nm steps using automatic exposure times. Averaged spectra were extracted from different image regions to allow a pHcalculation of both tablet layers. The ratios of the maxima were determined. Corresponding pHcalc values were calculated using a calibration curve of the fluorescence dye. Furthermore, pseudo-coloured fluorescence images were generated by separating the microacidity of the measured images using an acidic spectrum (assigned colour red) and a neutral spectrum (assigned colour green) of the spectra library. The measured spectrum of each data point was assigned to the closest matching spectrum. Therefore, acidic domains of the measured tablets appear red; areas with a pH>6 appear green within the pseudo-coloured images.

2.5. Micro acidity measurements using spatial spectral EPR imaging

2- and 3-layer tablets containing EPR spin probe AT (1 µmol/g powder) were used. Measurements were performed with a L-band EPR spectrometer (Magnettech GmbH, Berlin, Germany) using following parameters: B0-field 48.9 mT, scan range 8 mT, scan time per projection 30 s, modulation amplitude 0.1 mT, attenuation 6 dB, maximum gradient of 2.5 mT/cm, points per projections 1024, 31 projections/6 missing projections, image reconstruction giving an image matrix of 512 × 512 points and a spatial resolution of about 200 µm. The KSR/KSR-P layer of the tablet used for analysis was glued to a plastic bar which was placed into 100 mL of buffer pH 3 (see Section 2.3). The plastic bar with the affixed tablet was removed from the buffer at different time points. Adhering water on the surface of the tablet was removed carefully using absorbent paper before measuring. Two dimensional EPR images were collected for all tablet compositions after 10 min, 30 min, 1, 2, 3, 4 and 6 h of buffer contact. The pH of the buffer was analysed regularly and showed a stable pH of 3. The EPR spectra of the different image layers were extracted from the images. Only image domains with signal intensities over 30% were used for further analysis. The values of 2Δθe (distance 1st to 3rd peak) were determined from the extracted spectra. Resulting pHcalc values were obtained using a pH calibration curve of AT and plotted against the spatial position within the tablet. Experiments were performed in triplicate.

2.6. Influence of the microenvironmental pH on the drug release

Dissolution studies were carried out to investigate if different pH3 within a tablet could influence the drug release. Metformin-HCl and Ketoprofen were used as model drugs. Drug containing tablets were prepared by incorporating 17.4% of drug instead of lactose into the Kollidon SR layer giving a drug content of 34.8 mg per tablet (Table 1). The drug release was determined from 2-layer tablets B and C and 3-layer tablets E and F. Dissolution studies were carried out with an automatic dissolution tester (PTWS 310, Pharmatest Apparatebau, Hainburg, Germany) in 900 mL of buffer pH 3 (see Section 2.3) at 37 °C and 50 rpm. The drug release was analysed by measuring UV absorbance at 233 nm for Metformin-HCL and 275 nm for Ketoprofen and calculated using calibration curves. Dissolution experiments were carried out over 12 h and performed in triplicate.
2.7. Monitoring of hydration behaviour by means of NMR benchtop imaging

NMR imaging experiments were performed on a 3T-MRI spectrometer working at a frequency of 20 MHz and using a static magnetic field (B0) of 0.5 T (Maran DRX2, Oxford Instruments Molecular Biotools, Oxfordshire, UK). A standard spin-echo sequence was used with an echo time of 9.8 ms and a repetition time of 300 ms leading to an acquisition time of about 5 min for each image. Sixteen scans were accumulated to obtain 64 × 64 pixel images with a field of view of 4 cm2, which led to an in-plane resolution of 312.5 μm. 2- and 3-layer tablets were placed in a USP paddle dissolution apparatus with 900 ml of buffer pH 3 of 37 °C, stirred at 50 rpm, or in a beaker with 100 ml of same buffer at room temperature without stirring. The tablets were removed for MRI measurements after predefined time intervals and transferred to a sample holder. T1-weighted MRI images were measured after 10 min, 30 min, 1, 2, 3, 4 and 6 h of contact with buffer. Experiments were performed in triplicate. MRI intensity profiles of resulting images were investigated using Oxford Instruments Rimage V.O.NIX as plug-in for Image J.

3. Results

3.1. Microacidity measurements using a pH indicator dye

The microenvironmental pH of hydrated multi-layer tablets was visualised using the pH indicator bromocresol purple with a transition pH range of 5.2–6.8 and a colour change from yellow to purple. This dye was used to differentiate between tablet layers which assumed the pH of the surrounding buffer and areas with incorporated IBS [(H2O)2HPO4/citric acid]. The tablet layers with incorporated IBS were supposed to generate a pH5 of around 6 upon hydration while the pH of the surrounding buffer was 3 which enabled the monitoring of different colours depending on presence or absence of IBS. In addition, the colour change from yellow to purple could be easily monitored. Photographs of tablets A–F as whole and cross-sectioned after defined time intervals of contact with buffer are shown in Fig. 2. After 10 min of buffer contact, a differentiation between formulations A, B and C and D, E and F is easily possible. The HPMC-P and KSR-P layer of tablets A and D turned purple/blue immediately after contact with buffer, indicating a pH above 5. This finding corresponded to the expectation because the IBS was incorporated into both layers. The pH5 of the exterior region of the KSR-P layer changed to yellow after 2 h whereas the HPMC-P layer appeared mainly purple over more than 4 h. The HPMC layer of tablets B and E (without IBS) turned yellow after contact with buffer. In the case of tablet B, the HPMC layer changed into purple, indicating a pH4.5 above 5, after 30–60 min of buffer contact. In contrast, the HPMC layer of tablet E maintained a yellow/orange colour over the analysed time interval of 6 h. The KSR layer of tablets C and F (without IBS) turned yellow after contact with buffer. No obvious change in colour could be observed over the analysed time.

3.2. Microacidity measurements using multispectral fluorescence imaging

Multispectral fluorescence imaging of 2-layer tablets was accomplished to analyse the pH at the tablet surface by means of a hydrophilic fluorescence dye. The emission spectrum of this dye undergoes a pH-dependent wave length shift (Fig. 3(a)). pH5 values could be calculated independently from the intensities for a pH range from pH 5 to 8 using a calibration curve (Fig. 3(b); Schädlich et al., 2009). Pseudo-coloured fluorescence images and corresponding pH5 values of both tablet layers of tablets A, B and C are illustrated in Fig. 4. The pH5 of both layers of tablet A showed values between pH 6.5 and 7.5 over more than 6 h. Higher pH5 values were detected within the HPMC-P layer compared to the KSR-P layer. The pH5 of the HPMC layer of tablet B increased from a predominantly acidic environment below the dye detection limit of pH 5 to values above pH 6 after about 3 h of contact with buffer (Fig. 4(b)). The pH5 shifting was delayed in comparison with the pH indicator results. This observation can be explained by the hindered hydration of the tablets from only two dimensions (see Section 2.4). The pH5 of the KSR layer of tablet C remained below pH 5 over more than 6 h (Fig. 4(c)).

3.3. Microacidity measurements using spatial spectral EPR imaging

EPR imaging provides the possibility to obtain spatial information about the pH5 within the tablets non-invasively. For the investigation of the pH5 by EPR imaging, the stable nitroxide radical 4-Amino-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl (AT) was used as pH-sensitive spin probe. Protonation of pH-sensitive spin probes leads to changes in the spin density of the nitroxide group (Fig. 5(b)) and therewith associated changes in the EPR spectra.
Supplemental material

(d) Investigating alternative application fields of fluorescence imaging

Fig. 3. (a) pH-dependent wavelength shift of the emission spectrum of the fluorescence dye Carboxy SNARF®-1. A green and a yellow filter set were used. (b) pH sensitivity of the peak ratio of Carboxy SNARF®-1.

Fig. 4. (A–C) Mean pHM values of the surface of both layers of tablets A, B and C from one dimension at different time intervals of contact with buffer pH 3. No values could be determined for areas with a pH<5 (empty symbols). (a–c) Pseudo-coloured fluorescence images and corresponding schemata of tablets A, B and C after 30 min of buffer contact. Red (dark grey) domains symbolise dry and acidic regions (pH<5); green (light grey) domains symbolise a nearly neutral pHM (pH>6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Fig. 5. (a) EPR spectra (first derivates) of the spin probe 4-Amino-2,2,5,5-tetra-methyl-3-imidazoline-1-oxyl (AT) at different pH values. The dashed line symbolises $2a_N$ ($a_N$ = the hyperfine splitting constant) for the spectrum at pH 4. Note that the distance between the first and the third amplitude is larger for the nonprotonated form (pH 8). (b) Principle of pH sensitivity and calibration curve of AT.
Fig. 6. (a) EPR images of AT-containing tablet C at different time intervals of contact with buffer pH 3. (b) EPR spectra which were extracted from the images of: (1) the dry tablet, (2) a region within KSR-layer after 1 h of buffer contact, (3) a region within HPMC-P-layer of the same image, (4) relation of spectra (2) and (3) with specified $2\Delta H$. (c) Spatial and spectral cut of tablet C after 6 h of buffer contact.

Depending on pH (Fig. 5(a); Khramtsov et al., 1982). In particular, the distance of the first to the third peak ($2\Delta H$, where $\Delta H$ is the isotropic hyperfine splitting constant) changes with changing pH of the surrounding buffer. Thus, a quantification of pH is possible by means of a calibration curve of $2\Delta H$ against buffer pH (Kempe et al., 2010). The pH dependency of the EPR signal of the spin probe AT follows a sigmoid dependence (Fig. 5(b)). Therefore, the pH$_M$ calculation is only possible in a limited pH range of about ±1.5 pH units depending of the $pK_a$ of the spin probe ($pK_a$ of AT is 6.1). Other spin probes having different $pK_a$ values can be used to analyse different pH ranges.

Fig. 6(a) shows characteristic EPR images of tablet C at different time points of contact with buffer. The horizontal scale symbolises the spectral resolution (3 peaks of mobile AT) while the upright scale characterises the spatial resolution from the top to the bottom of the tablet (Fig. 6(c)). The dry tablet shows only one central peak of the immobile spin probe. Contact with buffer led to an increase in mobility of AT in the hydrated regions, visible through the appearance of the outer isotropic hyperfine splitting (Lurie and Mäder, 2005). The proportion of mobile to immobile spin probe increased steadily with time, detecting the liquid penetration to inner tablet regions which can be observed by the increase of intensity of the isotropic hyperfine splitting. It was also possible to follow the swelling process of the tablets because of the increase in spatial signal size of the images over time. The signals indicate a pH gradient within the wet tablet which is visible by the changing distance from first to third peak (both sloped downwards). EPR spectra were extracted out of the horizontal layers of the presented images. Fig. 6(b) shows typical EPR spectra of: (1) a dry tablet; (2) the KSR layer after 1 h of buffer contact and (3) the HPMC-P-layer of the same EPR image. By comparison of spectra (2) and (3), a changing distance of $2\Delta H$ can be found (4). $2\Delta H$ of spectrum 2 (KSR layer) overlay the calculable range, indicating a pH$_M$ below 4.5. The calculated pH value of spectrum 3 (HPMC layer) was 6.03 which can be explained by the presence of IBS within this layer.

Spatial resolved pH$_M$ values extracted from the EPR images of tablets A, B and C at different time intervals of buffer contact are presented in Fig. 7. The pH$_M$ of whole tablet A was found to be around pH 6 for over 6 h (Fig. 7(A)). The acidic pH$_M$ of the HPMC layer of tablet B increased quite fast to more neutral values above pH 6 (Fig. 7(B)) while the predominantly acidic pH$_M$ of the KSR layer of tablet C changed only marginally in the centre region of the tablet (Fig. 7(C)). EPR imaging experiments of tablets A–F were repeated using a citric acid/phosphate buffer of pH 5.5 to gain information of the influence of the pH of the surrounding buffer on the pH$_M$. Similar results concerning the formation of pH$_M$ gradients within tablets over time of buffer contact were obtained. Fig. 7(C2) shows the pH$_M$ gradients within tablet C during contact with buffer pH 5.5. Interestingly, the pH$_M$ of the KSR layer was below the pH of the surrounding buffer up to 1 h of buffer contact. A 10% Kollidon SR suspension in water generates a pH of about 4.6. Thus, Kollidon SR could cause the more acidic pH$_M$. After 1 h, the KSR layer assumed the pH of the surrounding buffer. The pH$_M$ increased to values above
3.4. Influence of the microenvironmental pH on the drug release

Dissolution studies of two model drugs were carried out to investigate the influence of the pH_{int} on the drug release. The anti-diabetic drug Metformin-HCl was used as freely soluble model drug showing pH-independent release behaviour. Primarily, the drug release from 2-layer tablets was analysed. Unfortunately, these tablets could not withstand the release conditions and both tablet layers separated after about 2 h of dissolution testing. The layer separation led to an increase in dissolution rate of Metformin-HCl caused by the increased diffusion area (Fig. 10(a)). An additional inter layer of glycerol monostearate could considerably enhance the integrity of the tablets and prevent the separation of both tablet layers over the analysed time interval. Fig. 10(b) demonstrates the release behaviour of Metformin-HCl from 3-layer tablet formulations E and F. The IBS was present in the KSR-P layer of tablet E; while none was present in the KSR layer of tablet F. As expected, no influence of the pH_{int} on the drug release of Metformin-HCl could be found.

In contrast, NSAID Ketoprofen was analysed as model drug showing a pH-dependent solubility. Ketoprofen is very slightly soluble at acidic pH (0.28 mg/ml at pH 4) and slightly soluble at pH 6.0 (3.68 mg/ml) (Sheng et al., 2006). The solubility increases with increasing pH because of the cumulative deprotonation of the carboxyl group (pK_{a} of 4.76). Fig. 10(c) demonstrates the dissolution profiles of Ketoprofen from 3-layer tablets E and F. Tablet formulation E enhanced the drug release considerably in comparison to tablet formulation F. The drug containing KSR-P layer of tablet E generated a pH_{int} of around 6, thus, leading to a higher solubility of Ketoprofen.
Supplemental material

(d) Investigating alternative application fields of fluorescence imaging


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started at the edges of the tablets but continued between the two layers, leading to a separation over time of hydration. Furthermore, diffusion processes between the layers could be facilitated. The swelling of the hydrated regions led to an increase in size. The tablets were completely hydrated after about 4 h of buffer contact. After 6 h, both layers were often separated. The additional interlayer of the 3-layer tablets prevented the penetration of water between the Kollidon SR and the HPMC layer. Therefore, the hydration process was slower because it continued only from the edges of the tablets to the inner regions. A dry core was existent even after 4 h of buffer contact. The 3-layer tablets remained complete over more than 6 h of hydration.

Characteristic T1-weighted BT-MRI images with corresponding intensity profiles of 2- and 3-layer tablets over time of buffer contact are presented in Fig. 12. Dark areas within the tablets refer to low spin densities and/or short relaxation times, which are related to dry parts of the tablets. Hydrated areas appear bright because of the water penetration and therewith associated increase in spin density. Relaxation times in the range of 10 up to hundred milliseconds give the brightest contrast under our measurement conditions (T1 weighted). The HPMC layer appears brighter than the Kollidon SR layer. HPMC forms a gel upon hydration. The water inside the gel layer was not as flexible as in the pores of the Kollidon SR matrix leading to shorter T1 relaxation times and a brighter signal which could also be confirmed by NMR relaxometry (data not shown). A swelling of the HPMC and Kollidon SR layer could be monitored by increase in size of the tablets. The different tablet formulations were exposed to two different hydration settings. Tablets exposed to USP dissolution conditions showed a faster water penetration into and erosion of the HPMC layer compared to the unstirred tablets, visible by a faster decrease in size. Water penetration between both layers of the 2-layer tablet C could be monitored after 30 min of buffer contact independent from used hydration setting which is also illustrated in the corresponding MRI intensity profiles. After 1 h, an additional central peak could be monitored within the intensity profiles (Fig. 12(b); C, 1 h). In contrast, the hydration of 3-layer tablets F proceeded only from the edges of the tablets caused by the aforementioned interference of the interlayer. The interlayer is clearly visible as black region between the HPMC and the KSR layer over the analysed time interval of 6 h. Because of its lipophilic character, almost no water penetrated into this region leading to low spin density and a black colour. The MRI intensity profiles illustrate the low signal intensity between the HPMC and the KSR layer even after 6 h of contact with buffer (Fig. 12(b); F, 6 h).

Fig. 12. (a) 1H NMR benchtop magnetic resonance image and corresponding signal intensity profile of tablet preparation F after 30 min of contact with buffer, exemplified for scale labelling of (b); (b) 1H NMR benchtop magnetic resonance images and corresponding signal intensity profiles of tablet preparations at different time intervals of contact with buffer. Cbeaker: 2-layer tablet C, hydration in unstirred beaker. CUSP: 2-layer tablet C, hydration in USP paddle dissolution apparatus at 50 rpm. Fbeaker: 3-layer tablet F, hydration in unstirred beaker. FUSP: 3-layer tablet F, hydration in USP paddle dissolution apparatus at 50 rpm. The arrow indicates the visible water penetration between both layers of tablet C.
4. Discussion

The purpose of present study was the detection of pHM gradients within multi-layer tablets. Different techniques were investigated for comparison purposes, all leading to similar results. A pH indicator dye was incorporated into tablets A–F, which allowed the differentiation between the tablet formulations because of their differences in local pH and therewith associated colour changes. Furthermore, it was possible to monitor the shifting of pHM between the tablet layers over time of buffer contact and to observe differences in the pHM of 2- and 3-layer tablets. Nevertheless, this technique allowed only a very rough determination of the pHM. It was rather difficult to relate a specified pH value to the colour grading of the indicator. Colours indicating same pH appeared different in both matrix forming excipients (HPMC and KSR). In addition, to investigate the pHM in the interior of the tablet, the tablet had to be cut. It was therefore not possible to analyse the pHM of one tablet continuously.

Fluorescence imaging gave the opportunity to calculate an average pHM of an estimated domain of each tablet layer using a fluorescence dye with pH dependent changes in the emission spectra. Similar pH gradients were detected compared to the results of the aforementioned method. However, a different hydration setting had to be used to allow a constant measuring area which changed and delayed the hydration process and made comparison with other results rather difficult. Higher pHM values were detected within the HPMC-P layer compared to the KSR-P layer of tablet A (same amount of IBS in both layers). The usage of different excipients could have an impact on the emission spectrum. The influence of the nature of excipient on the pHM calculation was therefore analysed by fluorescence imaging. Kollidon SR and HPMC showed no enhancement of fluorescence of IBS (pHM not shown). A 10% Kollidon SR suspension in water generates a pH around 4.6 which also influences the resulting pHM. Furthermore, the photographs of pH-indicator containing tablets showed a yellow discoloration of the surface of the previously blue KSR-P layer after 1–2 h (Fig. 2). In contrast, the colour of the HPMC-P layer changed only marginally. With fluorescence imaging, it was only possible to analyse the pHM of the surface of the tablets because of the limited penetration depth of the excitation and emission light. The pHM of the surface of the tablet could differ from those of the inner regions which can also contribute to the monitored differences.

Therefore, EPR imaging was accomplished to determine the spatial pHM distribution of the tablets non-invasively. EPR imaging provides the possibility to calculate the average pHM of hydrated inner and outer regions of different cylindrical layers of the tablet giving a spatial pHM resolution from top to bottom of the tablet. Although the analysis with this technique is more time-consuming, it gave unique information about the internal pH within analysed tablets and made a continuous measurement of one tablet over time of hydration possible. Furthermore, no influence of the nature of surrounding matrix material on the resulting 2D pHM values could be detected (data not shown). However, the pHM calculation is only possible in a limited pH interval of about ±1.5 pH units depending of the pHZC of the spin probe (pHZC of AT is 6.1). Therefore, no pHM values could be calculated in tablet regions showing a pHM below 4.5. It is possible to investigate the pHM within more acidic regions of the tablets using a spin probe with a lower pHZC.

The pH of the buffer strongly influenced the internal pH of tablet layers without IBS. Almost no influence could be monitored in the case of tablet layers with IBS (Fig. 7(C2)). The HPMC layer of tablet B (without IBS) showed an acidic pHM after contact with buffer but started to change to nearly neutral values after 30 min. After 2 h, the complete HPMC layer showed a pH below 4.5 (Fig. 7(B)). A possible reason could be the migration of IBS out of the KSR-P layer into the HPMC layer. In contrast, the HPMC layer of tablet E remained acidic over 6 h of buffer contact (Fig. 9(E)). The migration of IBS seems to be hindered by the lipophilic inter layer. In the case of the KSR layer of 2- and 3-layer tablet C and F (without IBS), an obvious pHM gradient over more than 6 h of buffer contact was determined. Especially in the case of tablet F, the pHM of the KSR layer showed an acidic pH of ±4.5 over the analysed time interval of 6 h, confirming the protective character of the inter layer (Fig. 9(F)). This finding could also be valuable to separate drugs with different pH stability optima by the usage of multi-layer tablets with an additional lipophilic inter layer. However, the pHM of the KSR layer of tablet C increased only marginally in the centre region of the tablet as well, which was different from the behaviour of the HPMC layer of tablet B. The different behaviour of both matrix-forming excipients might possibly be caused by a faster water exchange within the KSR layer in comparison to the HPMC layer. Furthermore, the acidic behaviour of Kollidon SR seems to have an influence on the pHM generation as well.

Dissolution studies were carried out to analyse the influence of the pHM on the drug release. Two model drugs were incorporated into the KSR/KSR-P layer of tablet E/F (with and without IBS). Metformin–HCl shows a pH independent solubility. Therefore, both formulations showed same drug release (Fig. 10(b)). In contrast, the release of Ketoprofen could be modified by the incorporation of the IBS. Ketoprofen shows a pH dependent solubility with an improved solubility under neutral conditions (see Section 3.4). Therefore, the drug release of tablets E and F (contains HPMC in the KSR-P layer) was not comparable to formulation F (without IBS) (Fig. 10(c)). These finding is in agreement with literature data where the drug release of weak acids could be improved by the incorporation of alkaline excipients (Doherty and York, 1989; Riis et al., 2007; Tran et al., 2008). Further formulation optimization would be needed for a sustained drug release over 12 h. This issue was beyond the scope of this work as it was intended to keep the formulation of the layers constant for comparability purposes.

Benchtop NMR imaging was accomplished to further analyse the differences in the hydration behaviour of 2- and 3-layer tablets. The MRI signal of the HPMC layer appeared brighter than compared to the signal of the KSR layer. The water inside the gel layer of HPMC is not as flexible as in the pores of the KSR matrix leading to a shorter T1 relaxation time and a brighter signal. This issue could also have an influence on the different behaviour regarding the migration of IBS. Besides, it could be detected that water penetrated between the two layers of the 2-layer tablets (Fig. 12(C)) which could enable a fast migration of IBS from the KSR-P to the HPMC layer of tablet B. Furthermore, a separation of both layers could be facilitated. The water penetration could be prevented by an additional lipophilic inter layer which improved the integrity of the tablets and possibly hindered the migration process of the IBS (Fig. 12(F)). In addition, 2- and 3-layer tablets were exposed to two different hydration settings. Tablets exposed to USP dissolution conditions showed a faster water penetration into and erosion of the HPMC layer compared to the unstirred tablets (Fig. 12). These findings are consistent with previous work, showing the dependence of erosion and hydration processes of hydrogel-forming HPMC on mechanical stress (Costa and Lobo, 2001; Kavanagh and Corrigan, 2004). Further studies have to be carried out to investigate, if mechanical stress could also change the migration behaviour of the IBS.

In conclusion, pHM gradients within multi-layer tablets could be analysed by 3 different techniques, in particular, a pH indicator dye, fluorescence imaging and EPR imaging. It was possible to gain information about the pHM with all applied techniques. The qualitative results were similar but the informative value showed major differences. The incorporation of a pH indicator dye turned out to be a simple, fast and inexpensive method to get an overview over proceeding processes. However, no precise pHM determina-
position was possible and the inner tablet regions could be analysed only invasively. Fluorescence imaging produced calculable results of the pHi of the tablet surface. A spatial distribution of the surface pHi could be provided. However, a different hydration setting had to be used, expicit interactions were hard to predict and the inner regions of the tablet can be analysed only by cutting the tablet. EPR imaging proved to be a powerful tool for the determination of spatial pHi information non-invasively. However, it should be emphasized in hydration dosing that pHi describe an average pHi value of a thin tablet layer, possibly forming a pHi gradient inside this layer with different pHi values in the outer regions compared to the centre of the tablet. Furthermore, it is a time consuming method which requires expensive equipment. Nevertheless, because of its superior advantages, EPR imaging was used as a fully developed tool for in vivo analysis. The number of independent variables on the pHi was investigated. The incorporation of an IBS strongly influenced the pHi as well as the nature of used matrix forming excipient. Kollidon SR generated a more acidic microenvironment compared to HPMCC, which was obvious in particular when buffer pH 5.5 was used where the pHi of the KSR layer underlay the buffer pH. The pHi of the KSR layer maintained acidic over the analysed time interval. Otherwise, the HPMCC layer was able to keep primary acid pHi to more neutral values although the acidic properties of the surrounding buffer which may be caused by the migration of IBS from the KSR-P layer. The variation of the buffer pH had an influence on the pHi especially within tablet layers without IBS. An additional lipophilic inter layer strongly improved the integrity of both layers. Furthermore, it acted as pH neutral region which could decrease diffusion processes between the layers and therefore influence the pH gradient process. BT-MRI was accomplished to gain a deeper insight on the differences of proceeding processes during hydration of 2- and 3-layer tablets. The progressive characteristic of the inter layer was confirmed which could prevent water penetration between the HPMCC and the KSR layer, leading to the aforementioned advantages. Mechanical stress influenced the hydration process as well, which was monitored using different hydration settings. Moreover, an influence of the pHi on the drug release curve by acid analysis. The intensity of differences variables on the pHi was investigated. The drug release of Metformin-HCl, showing pH independent solubility, was not influenced by varied pH's as expected.

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References


(d) Investigating alternative application fields of fluorescence imaging


Annex

Acknowledgements .............................................................. I

Publication list ....................................................................... III

Curriculum vitae .................................................................... VII

Declaration of the self-contribution of research articles ........ VIII

Selbstständigkeitserklärung ................................................ XI
First and foremost, with a deep sense of respect and heartfelt gratitude I would like to extend my unreserved indebtedness to my supervisor, Prof. Dr. habil. Karsten Mäder, head of the Pharmaceutical Technology Group within the Department of Pharmaceutical Technology and Biopharmaceutics at the Martin Luther University of Halle-Wittenberg in Germany. I want to thank him for the opportunity to join his research group and for the suggestion of the very interesting topic of my PhD work. Furthermore I would like to thank him for all kinds of scientific discussions, questions and inspirations while providing me freedom immersing into the fluorescence imaging research.

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Publication list

Research articles (published or under peer review)

(a) Investigation of the in vivo fate of a water soluble polymer


(b) Characterization of nanocarriers and their potential usage in cancer therapy


(c) Characterization of in situ forming implants for potential controlled API release


(d) Investigating alternative application fields of fluorescence imaging


Research articles (in preparation)

(d) Investigating alternative application fields of fluorescence imaging

(IX) Li, J., Schädlich, A., Hause, G., Vogel, J., Kuntsche, J., Groth, T., Mäder, K., Pre-clinical in vivo studies of oily core PEG-PLGA nanocapsules using fluorescence imaging. (in preparation)

(X) Lochmann, A., Schädlich, A., Nitzsche, H., Metz, H., Schön, I., Schwarz, E., Mäder, K., Quantitative monitoring of the in vivo efficiency of rhBMP-2 loaded PLGA and PEG-PLGA microparticles by means of optical imaging, CT and BT-MRI. (in preparation)
Conference contributions (selection)

(a) Investigation of the *in vivo* fate of a water soluble polymer


(b) Characterization of nanocarriers and their potential usage in cancer therapy


(c) Characterization of *in situ* forming implants for potential controlled API release

7. **Schädlich, A.**, Kempe, S., Ullrich, S., Mäder,K., (2010) *In vitro* and *in vivo* pH measurement studies of *in situ* forming sucrose ester and PLGA implants using EPR spectroscopy and fluorescence imaging. 7th APV World Meeting, Malta
(d) Investigating alternative application fields of fluorescence imaging


Curriculum vitae

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sgd. A. Schädlich
Dipl. Pharm. Andreas Schädlich
Declaration of the self-contribution of research articles

The cumulative doctoral thesis is compiled with several research articles published or in peer-review. Most of the research work in the thesis was carried out by myself independently in the Pharmaceutical Technology Group at the Martin Luther University under the supervision of Prof. Dr. habil. K. Mäder. His contribution to the publications includes the strategic planning of the experiments and also the final review of the articles. In addition Prof. Dr. habil. K. Mäder supported the published research work in many scientific discussions, questions and inspirations.

(a) Investigation of the in vivo fate of a water soluble polymer


My contribution was about 65%. I have planned and performed all in vivo and ex vivo experiments. Prof. Dr. habil. J. Kressler has supervised his Mr. T. Naolou and Dr. E. Amado who have performed the labeling and Dr. R. Schöps in performing in vitro filtration experiments.

I drafted the manuscript and completed the publication after discussing it with the co-authors.


My contribution was about 25%. I made all in vivo and ex vivo fluorescence imaging experiments and I have written the related paragraphs in the manuscript.

My contribution was about 70 %. I made all *in vivo* and *ex vivo* fluorescence imaging experiments and I have written the manuscript.

(b) **Characterization of nanocarriers and their potential usage in cancer therapy**


My contribution to both papers was about 70 % respectively. All biodistribution studies and the writing of the manuscripts were carried out by myself. The preparation of the nanoparticles was performed by Dr. C. Rose, member of Prof. Dr. A. Göpferich’s group at the University of Regensburg. Associate Prof. Dr. J. Kuntsche carried out the particle size measurements and mainly reviewed the article.s F. Tenambergen also supported the particle size measurements. Dr. H. Caysa and her group leader Dr. T. Müller have supported the *in vivo* experiments while providing the xenograft tumor model, performing the nanoparticle injections and supporting the cell experiments.

My contribution was about 80%. All studies were mainly performed by myself. I have written the manuscript and completed it after discussing the paper with the co-authors. S. Hoffmann, Dr. H. Caysa and Dr. T. Müller have partly supported the *in vivo* experiments. The preparation of the nanoparticles/nanocapsules was performed by Dr. C. Rose and Dr. J. Li. Associate Prof. Dr. J. Kuntsche carried out the particle size measurements and reviewed the article. Prof. Dr. A. Göpferich supervised the nanoparticle preparation and reviewed the manuscript.

(c) **Characterization of *in situ* forming implants for potential controlled API release**


My contribution was about 75%. All fluorescence imaging as well as the writing of the manuscript was performed by myself. Dr. S. Kempe from the same group has carried out the EPR experiments and has revised the manuscript.

(d) **Investigating alternative application fields of fluorescence imaging**


My contribution was about 30%. I have performed the fluorescence pH measurements and have written the related paragraphs in the manuscript.

Halle (Saale), den 24. September 2013

sgd. A. Schädlich  
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sgd. Karsten Mäder  
Prof. Dr. habil. Karsten Mäder  
(Co-Author)
Selbstständigkeitserklärung

Hiermit erkläre ich gemäß § 5 der Promotionsordnung der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg, dass ich die Ergebnisse der vorliegenden kumulativen Dissertationsarbeit

Pre-clinical in vivo studies of parenteral drug delivery systems using non-invasive multispectral fluorescence imaging

am Institut für Pharmazie im Institutsbereich Pharmazeutische Technologie und Biopharmazie der Martin-Luther-Universität Halle-Wittenberg unter Anleitung von Herrn Professor Dr. rer. nat. habil. Karsten Mäder selbstständig erarbeitet und die Dissertation ohne fremde Hilfe verfasst habe.

Ferner erkläre ich, dass ich keine anderen als die von mir angegebenen Quellen oder Hilfsmittel benutzt habe und die den verwendeten Werken wörtlich- oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Weiterhin erkläre ich, dass ich mich mit der vorliegenden Dissertationsarbeit erstmals um die Erlangung eines Doktorgrades bewerbe.

Halle (Saale), den 24. September 2013

sgd. A. Schädlich

Dipl. Pharm. Andreas Schädlich
Das Schönste, was wir entdecken können, ist das Geheimnisvolle.

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science.

He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein (1879 - 1955)

Cover picture: Unmixed fluorescence image of a female mouse 24 hours after i.p. injection of PVA. The incremental jet color image represent the threshold fluorescence PVA-TMR signal. Reprinted and adapted from Scientifically Speaking News (Controlled Release Society) 27 (2), Cover page, Tracking the in vivo fate of high molar mass poly(vinyl alcohol) using multispectral fluorescence in vivo imaging, Andreas Schädlich, Yanjiao Jiang, Jörg Kressler and Karsten Mäder. © Copyright 2010, with permission from Controlled Release Society.

Back: Unmixed fluorescence image of red currants