Aus dem Institut für Tierpathologie
des Fachbereichs Veterinärmedizin der Freie Universität Berlin

Anti-Inflammatory Dendritic Polyglycerol Sulfate Nanoparticle:

Biodistribution, Elimination, Cellular Localisation and Toxicopathology in Mice

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Dedicated to

my parents

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List of Abbreviations

Declaration of Originality / Selbständigkeitserklärung

List of Abbreviations

ADME	**A**bsorption, **d**istribution, **m**etabolism, **e**xcretion
apo A-1	**Apo**lipoprotein **A-1**
aPTT	**A**ctivated **p**artial **t**hromboplastin **t**ime
BBB	**B**lood-**b**rain **b**arrier
CIA	**C**ollagen-**i**nduced **a**rthritis
cpm	**C**ounts **p**er **m**inute
DAPI	4',6-**dia**midino-2-**p**heny**l**indole
DB	**D**egree of **b**ranching
DLS	**D**ynamic **l**ight **s**cattering
DS	**D**egree of **s**ulfation
dPGS	**d**endritic **p**oly**g**lycerol **s**ulfate
EC	**E**ndothelial **c**ell
EDX	**E**nergy **d**ispersive (**x**-ray) spectroscopy
EELS	**E**lectron **e**nergy **l**oss **s**pectroscopy
EMA	**E**uropean **M**edicines **A**gency
FDA	**F**ood and **D**rug **A**dministration
FFPE	**F**ormalin-**f**ixed, **p**araffin-**e**mbedded
HD	**H**ydrodynamic **d**iameter
HDL	**H**igh-**d**ensity **l**ipoprotein
HE	**H**aematoxylin **e**osin
ICC	**I**ndo**c**arbo**c**yanine
ICH	**I**nternational **C**ouncil for **H**armonisation of Technical Requirements for Pharmaceuticals for Human Use
IHC	**I**mmuno**h**isto**c**hemistry
i.v.	**I**ntra**v**enous
LC	**L**euco**c**yte
LD50	**L**ethal **d**ose, **50**%
LMA	**L**ight **m**icroscopic **a**utoradiography
LSC	**L**iquid **s**cintillation **c**ounting
LSM	**L**aser **s**canning **m**icroscopy
MARG	**M**icro**a**uto**r**adio**g**raphy
MPS	**M**ononuclear **p**hagocyte **s**ystem
MRI	**M**agnetic **r**esonance **i**maging
NHS	**N**-**h**ydroxy**s**uccinimide
NIR	**N**ear **i**nfra**r**ed
NM	**N**ano**m**aterial

NP	**N**ano**p**article
PALM	**P**hoto**a**ctivated **l**ocalisation **m**icroscopy
PBS	**P**hosphate **b**uffered **s**aline
PEG	**P**oly(**e**thylene)**g**lycol
PG	**P**oly**g**lycerol
p.i.	**P**ost **i**njection
PMT	**P**hoto**m**ultiplier **T**ube
PSGL-1	**P**-**s**electin **g**lycoprotein **l**igand-**1**
QWBA	**Q**uantitative **w**hole **b**ody **a**utoradioluminography
QWOA	**Q**uantitative **w**hole **o**rgan **a**utoradioluminography
RES	**R**eticulo**e**ndothelial **s**ystem
SEM	**S**canning **e**lectron **m**icroscopy
s.c.	**S**ub**c**utaneous
SLeX	**S**ialyl **L**ewis **X**
SRB1	**S**cavenger **r**eceptor **B1**
STED	**St**imulated **e**mission **d**epletion microscopy
STEM	**S**canning **t**ransmission **e**lectron **m**icroscopy
STORM	**St**ochastic **o**ptical **r**econstruction **m**icroscopy
TEM	**T**ransmission **e**lectron **m**icroscopy
TT	**T**hrombin **t**ime
TyS	**Ty**ro**s**ine
WBA	**W**hole **b**ody **a**utoradiography
WOA	**W**hole **o**rgan **a**utoradiography

x

1   INTRODUCTION

"Nanotechnology has the potential to create major technological breakthroughs" (European Commission Press Release 2012).

Nanoscale particles, e.g. fullerenes, naturally emerge from combustion and volcano eruption or occur, in living organisms, e.g. as biogenic magnetite. In the course of the industrial revolution, anthropogenic induced nanomaterials (NMs) started to be produced in high numbers mainly as side products, with only a few being intentionally designed (Oberdörster et al. 2005). In recent years, however, new classes of nanomaterial-related applications have been developed in vast numbers. The physicochemical properties of these new NMs can be engineered precisely to meet the requirements for the intended application, thus generating a nearly unlimited diversity of materials (Buzea et al. 2007; Roduner 2006).

At present, NMs are already contained in a variety of products such as UV filters in sun screens (Buzea et al. 2007; Hoet et al. 2004) and antiperspirant textiles as well as in technical applications such as flat panel displays, lithium-ion batteries or solar cells (Buzea et al. 2007). Furthermore, biomedical applications of NMs are an area of considerable research especially with a view to bioanalysis, biosensors, medical devices, drug delivery, and therapeutic applications (Mazzola 2003). Using NMs for drug delivery facilitates high tissue concentrations of a compound and/or enhances tissue-specific recognition. This is of immense interest because a controlled release at the affected site reduces the amount of a drug required and prevents adverse effects in tissues other than that of the target (Slowing et al. 2008). Application of NMs in diagnostics includes their use as contrast agents, currently mainly for magnetic resonance imaging on the basis of metals or as carriers for dyes (Mazzola 2003).

Due to their remarkably small size, in the billionth of a meter range, NMs exhibit different or additional properties compared to their bulk material (Rivera Gil et al. 2010). Some of these properties can be derived from their macroscale precursor, but others change drastically when the NM falls below a certain size. Notably, the size of NMs lies within the dimensional range of biological components and little is known about their biological and environmental impact. In both of these respects NMs will have to be scrutinised regarding their safety and long-term effects as their use becomes more prevalent (Wiesenthal et al. 2011). The ability to design a large variety of different NMs, whose behaviour often cannot be predicted, consequently leads to a huge body of work (Rivera Gil et al. 2010).

While *in vitro* studies provide basic insights on the cytotoxicity of NMs or their mechanisms of action, only *in vivo* studies offer the opportunity to understand the relationship between the physicochemical properties of NMs and their unique biological effects (Li and Chen 2011). To date, systematically determined information on this interrelation is too inadequate to predict the safety of a NM for the use in humans. Without sufficient systematic knowledge the biological effects such as distribution, sites of accumulation and toxicological profiles have to be investigated for each NM individually. In addition, methods for the detection of NMs *in vivo* that leave the NMs chemical structure untouched and deliver unadulterated data are limited (Buzea et al. 2007; Grainger 2009). The study presented here introduces such a method and provides a general understanding of the biological impact of the anti-inflammatory nanoparticle (NP) dendritic polyglycerol sulfate (dPGS) on the whole living organism of mice. This understanding is crucial for future systematic investigations towards a therapeutic and diagnostic agent.

2   LITERATURE

2.1   Nanomaterials and Nanoparticles

The term nano ([nanos] from the Greek word for "dwarf") generally refers to materials with dimensions in the size range of nanometres (nm), with 100 nm frequently used as the cut-off size to differentiate the nanoscale from the microscale. By definition NMs must have at least one dimension with less than 100 nm, while NPs are spherical and have three external dimensions in the nanoscale (ISO/TS 80004-2:2015). Nevertheless, materials probably with dimensions up to 500 nm and therefore outside the 100 nm range also exhibit properties or phenomena attributable to their dimension, such as translocation through biological barriers (Kendall and Holgate 2012; Oberdörster et al. 2009). For regulatory considerations, the Food and Drug Agency (FDA) as well as the European Medicines Agency (EMA) even consider products up to an upper limit of 1,000 nm that are engineered to show dimension-dependent chemical or physical properties or biological effects as products involving nanotechnology (FDA 2014).

NPs can be divided according to their shape, size, surface charge and physicochemical properties (Papakostas et al. 2011) or are classified as either "hard" (i.e. inorganic-based) or "soft" (i.e. organic-based; Tomalia 2009). Hard NPs have conducting or semiconducting properties and tend to form crystalline or 3D lattices that keep their shape and size during processing or when interacting with biological systems. This class includes metals, semi-metals, metal chalcogenides and oxides as well as carbon allotropes containing a conjugated-system. Soft NPs are more flexible and exhibit non-conducting, insulator-type properties associated with covalent assemblies and an amorphous, non-crystalline behaviour. They are composed of materials, many of which are found in biologic systems including carbon, hydrogen, oxygen, nitrogen, etc. (Li and Chen 2011; Tomalia 2009).

Due to their small size, NPs have a large area-to-mass ratio leading to an increased surface or interface area for chemical reactions (Buzea et al. 2007). Furthermore, surface atoms have a lower coordination number (i.e. fewer neighbours) and are thus less stabilised. Accordingly, the reactivity of the particles is increased (Roduner 2006). Moreover, at the nanoscale quantum effects are not superimposed by other phenomena and thus dominate the behaviour and characteristics of a particle affecting its optical, electric and magnetic properties (UK Royal Society and Royal Academy of Engineering 2004). Fluorescence, ionisation potentials, electronic conductivity, magnetic permeability and other characteristics become size-dependent; this is caused by the wave nature of the matter and the dimension of the material (Roduner 2006; Tomalia 2009; UK Royal Society

and Royal Academy of Engineering 2004). Examples of materials that already utilise these effects include quantum dots and quantum well lasers for optoelectronics (UK Royal Society and Royal Academy of Engineering 2004).

Furthermore, during particle synthesis various parameters, including size, shape and surface charge, can be adjusted to fine-tune the properties of interest (Mazzola 2003; Roduner 2006). This ability to precisely engineer materials has a huge potential to innovate many areas of technology and satisfy as yet unmet needs in biomedicine. Potential applications in biomedicine range from *in vitro* diagnostics to *in vivo* imaging, biomedical devices, therapy, or delivery of drugs (Almeida et al. 2011; Mazzola 2003). It is therefore no surprise that the European Commission has identified nanotechnology as a key enabling technology that provides the basis for further innovation and new products (European Commission Recommendation 2011/696/EU, OJ L 275, 20.10.2011).

The different or additional chemical and physical properties of NPs are in many cases not predictable with the current knowledge (Rivera Gil et al. 2010). Since biological and chemical reactions often take place at the surface of a material and the size of NPs ranges within the dimensions of biological components, they have the potential to migrate easily into biological systems and interact with cells (Salata 2004). These features have raised concerns about their impact on health as well as the environment and have generated the field of nanotoxicology, which aims to address the safety issues of engineered NPs (Lynch and Dawson 2008; Rivera Gil et al. 2010).

One of the major challenges remaining in the characterisation of the biological impact of NPs is to establish a common accepted framework with standard operating protocols for a systematic analysis, in order to derive general rules to predict the interrelation of physicochemical NP properties and their biological effects (Fadeel et al. 2015; Grainger 2009). In the field of biomedicine, the lack of consensus and the hitherto fragmentary state of knowledge regarding the methods best suited for safety and efficacy testing complicates preclinical safety assessments (Grainger 2009). Without understanding the correlation of the physicochemical properties of the particles and their biological impact, each material with any morphology has to be studied for adverse effects individually (Buzea et al. 2007).

2.2   Safety of Nanoparticles for Biomedical Applications

The safe handling and application of NPs in biomedicine will fundamentally depend on how the authorities in Europe, the United States of America (USA) and elsewhere will regulate the approval of products containing NMs in the future. One pioneer in developing models for safety and efficacy assessment is often the FDA in the USA. Each medicinal product approval includes the preclinical, clinical and post marketing phase on a case-by-case basis (Choi and Frangioni 2010; Eifler and Thaxton 2011). The FDA considers this approach an adaptable, product-specific policy sufficient for NM-containing products (FDA 2014) and has decided, for now, to regulate drugs containing NMs in accordance with the legal standard of the respective product type, e.g. intravenously administered drugs (Bawa 2013; FDA 2014; Hamburg 2012). A similar approach has been chosen by the EMA, as published in their reflection paper on nanotechnology-based medicinal products for human use (EMA 2006).

This means NPs in biomedical products are currently not subject to additional regulatory requirements (Tyner and Sadrieh 2011). However, the Nanotechnology Task Force of the FDA as well as the EMA take into account the specific behaviour of NMs and recommend a staged approach, which is based on scientific progress or on experience with the evaluation procedures of marketing authorisation applications, to determine whether current tests are adequate to support risk management decisions (EMA 2006; Hamburg 2012). For generic, iron-containing NPs (EMA/CHMP/SWP/620008/2012) and coated NPs (EMA/325027/2013), for example, the EMA has published specific reflection papers. With the nanotechnology field growing, further specific guidance is expected to follow.

With regard to this particular study, the requirements for preclinical safety assessment are outlined in more detail: NMs beyond the scope of the specific guidance documents referenced will be assessed in accordance with the guidance on non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals, M3(R2) for "regular" small molecule drugs (i.e. pharmaceuticals) of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) which is mandatory in the USA, Europe and Japan (ICH 2009).

According to the guidance document M3(R2) the preclinical safety assessment includes pharmacology, toxicokinetic and pharmacokinetic studies, as well as toxicity studies including general, reproductive and genetic toxicology (ICH 2009). In kinetic studies, the interaction of a compound with the organism is evaluated by its absorption, distribution, metabolism, and excretion (ADME). These parameters are important for the interpretation of pharmacological (efficacy) and toxicological (safety) findings because they provide essential information on the relation between the tissue distribution and accumulation at the potential sites of action. Subsequently, this information can be used to develop a rational study design (ICH 1994).

Besides the tests listed above for regulatory submission, NP approval may need other parameters. For example, in addition to the standard physiochemical information, those parameters related to their unique features, such as particle size, particle shape (morphology), particle distribution, surface effects (including surface area, reactivity, and coatings) and aggregation/agglomeration effects in relevant systems, might be required. All these may affect the kinetics and subsequently the safety and efficacy of an NP (Tyner and Sadrieh 2011; Zolnik and Sadrieh 2009). Furthermore, some NPs may interfere with standard tests, such as dendrimers with endotoxin tests (Zolnik and Sadrieh 2009). This means standard tests require verification when dealing with NPs. For purity, stability, and general batch-to-batch variability control, additional product-specific testing and manufacturing monitoring might be necessary (Hamburg 2012).

This study surveys the fundamental safety aspects of the NP dendritic polyglycerol sulfate (dPGS). More precisely, it determines the distribution, cellular localisation, excretion and pathomorphological changes for two routes of application.

2.3   DPGS Nanoparticles for Biomedical Applications

In 2004 *in vitro* studies by Türk and co-workers showed that sulfated polyglycerol NPs, referred to as dendritic polyglycerol sulfate (dPGS), prolonged the activated partial thromboplastin time (aPTT) and the thrombin time (TT) and inhibited both the classical and alternative complement activation. The anticoagulant activity was found to be comparable to that of heparin, whereas complement activation was even more efficiently inhibited (Calderón et al. 2010; Türk et al. 2004). A study of Shaunak and co-workers, also published in 2004, claimed that polyanionic dendritic structures possess anti-inflammatory properties *per se* (Shaunak et al. 2004). Based on this assumption, Dernedde and co-workers investigated dPGS regarding its anti-inflammatory properties and found that the anti-inflammatory mechanism of dPGS is based on an inhibition of leucocyte trafficking (Dernedde et al. 2010).

Leucocytes are part of the immune system that defends higher organisms against microbes and noxious substances. In the case of an infection, they migrate from the blood vessels to the infected tissue promoting inflammation to eliminate the agent (Woelke et al. 2013). In some instances, the immune response can, however, be detrimental and feature the pathogenesis of a disease. Suppressing leucocyte extravasation is a promising approach to prevent diseases that are related to over exuberant activity or misdirected inflammatory responses such as rheumatoid arthritis or psoriasis (Chavakis et al. 2009; Luster et al. 2005)

2.3.1   Mode of Anti-inflammatory Action of dPGS

The polyanionic dPGS particle blocks the interaction of the cell adhesion molecules P-selectin and L-selectin and their ligands (Figure 1), presumably by occupying the binding sites of the selectins through electrostatic interaction (Dernedde et al. 2010) thereby preventing leucocyte extravasation.

L-selectin is present on most leucocytes, whereas P-selectin is stored in secretory granules of platelets and endothelial cells. E-selectin is inducibly expressed by inflamed endothelial cells (Kneuer et al. 2006; Ley et al. 2007). The interaction of selectins with their natural ligands (Woelke et al. 2013), of which only P-selectin glycoprotein ligand-1 (PSGL-1) is characterised comprehensively (Ley 2003), is part of the leucocyte adhesion cascade (Woelke et al. 2013). This cascade includes the events tethering/capture, rolling, slow rolling, activation and firm adhesion/arrest of the leucocytes to the blood vessel wall (Ley et al. 2007) and is composed of interlocked, sequential steps, where each step is the prerequisite for the next (Luster et al. 2005). A preparatory step for the initiation of the cascade is a changed blood rheology, as triggered by inflammatory associated vasodilation, which forces the leucocytes to the vessel periphery (Ley et al. 2007). Particularly efficient at mediating capture and rolling are L-selectin and P-selectin (Luster at al 2005). L-selectin can also facilitate secondary leucocyte capture by leucocyte-leucocyte interaction and thus has a key role in this process. (Ley et al 2007). P-selectin additionally works together with E-selectin in mediating initial leucocyte adhesion (Kneuer et al. 2006). E-selectin also stabilises rolling. Rolling leucocytes can be activated by chemokines leading to conformation changes of leucocyte integrins and thereby mediating firm adhesion (Chavakis et al. 2009; Luster et al. 2005). This adhesion is crucial for the transmigration of the leucocytes; firmly adhered leucocytes, "crawl" over the endothelial surface and emigrate when they reach cell junctions or an appropriate area for transcellular migration (Chavakis et al. 2009; Luster et al. 2005). Besides leucocyte extravasation, tumour cell metastasis is probably fostered by selectin interaction (Barthel et al. 2007; Varki and Varki 2001). Because the selectin interactions have overlapping functions, targeting only one selectin can be insufficient to prevent extravasation (Jung and Ley 1999).

2.3.1.1   Interaction of dPGS with Selectins

All selectins possess a C-type lectin domain at the N-terminus. This domain comprises a calcium-binding pocket, consisting of a calcium ion (Ca^{2+}) ligated by three amino acids (Bouyain et al. 2001; Woelke et al. 2013). The natural ligand of the selectins, PSGL-1, contains two binding epitopes for the interaction with selectins; a sialyl Lewis X (SLeX) motif and a polypeptide part including three post-translationally sulfated tyrosines (TyS) near the N-terminus (Woelke et al. 2013). It is assumed that SLeX non-covalently binds the three

acidic amino acids of the calcium-binding pocket of the selectins, and/or Ca^{2+} itself. The calcium-binding pocket of the selectins is mostly conserved in sequence and structure (only one of the three amino acids differs between the selectins), whereas the interaction of the selectins with the peptidic part of PSGL-1 strongly depends on the selectin structure.
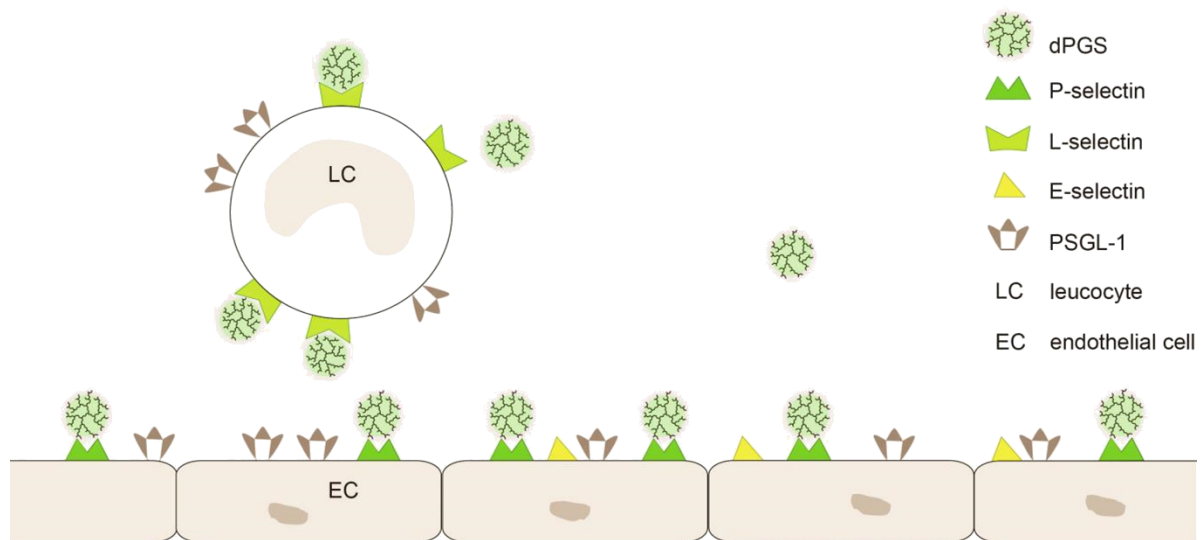
Figure 1: Principle sketch of dPGS interaction with the selectins P and L. Modified from Dernedde et al. 2010. The polymer shields L-selectin and P-selectin from their natural ligands and prevents leucocyte extravasation

Selectins are mainly positively charged, except for E-selectin that has a neutral net charge, while the PSGL-1 peptidic part carries a negative net charge, formed by the TySs mentioned above, which are located at residues 46, 48 and 51. These three TySs form salt-bridges with positively charged amino acids of the selectins (Woelke et al. 2013). As an exception in the family of selectins, the modelled structure of E-selectin shows no salt-bridge partner for TyS48 and a neutrally charged glutamine for TyS51. The authors conclude that due to its charge neutrality, E-selectin does bind the SLeX saccharide but is incapable of long-range attractive electrostatic interactions with the peptidic part of the PSGL-1 ligand (Woelke et al. 2013).

In general, the interaction of selectins with their ligands is dominated by electrostatic interrelations. The difference in charge and the reduced ability of E-selectin to form salt-bridges with the negative, sulfated TySs of PSGL-1 may explain why dPGS fails to block E-selectin ligand interaction (Dernedde et al. 2010; Weinhart et al. 2011b).

The negative charge of dPGS is defined by its degree of sulfation (DS). Besides the negative charge the special ligand presentation of dPGS, including the core size and the degree of branching (DB), influences the efficacy of the interaction with selectins. The dependence on the ligand presentation illustrates the supposed multivalent nature of dPGS (Dernedde et al. 2010; Paulus et al. 2014; Weinhart et al. 2011b). The multivalency might explain why, in contrast to other approaches for the inhibition of selectin-mediated adhesion (anti-selectin antibodies, inhibitors based on SLeX and its isomers), dPGS also exhibits a high binding affinity *in vivo* (Dernedde et al. 2010). This makes dPGS attractive for pharmaceutical utilisation both as a diagnostic marker for selectins and as a therapeutic drug, the latter either in the form of an anti-inflammatory agent itself or due to its potential as a carrier for anti-inflammatory drugs (Mazzola 2003). Besides modulating inflammation, the selectin-blocking properties of dPGS may also be able to suppress metastasis in anti-cancer therapy (Barthel et al. 2007; Varki and Varki 2001)

2.3.2   Dendritic Polyglycerol - Between Dendrimers and Hyperbranched Polymers

Dendritic polyglycerol sulfate is based on polyglycerol (PG). The term 'dendritic' ('tree-like') is used to indicate the affiliation to a family of structurally branched molecules covering dendrons, dendronised polymers, perfect dendrimers and hyperbranched polymers, where polyglycerol is located between perfectly branched dendrons/dendrimers and hyperbranched polymers (Figure 2; Quadir and Haag 2012).

dendron   perfect dendrimer   dendritic polyglycerol   hyperbranched polymer

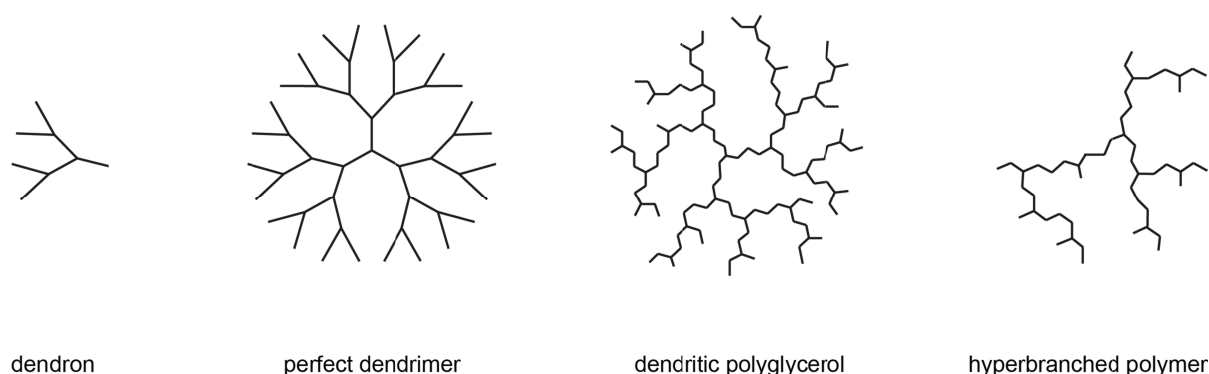Figure 2: Schematic comparison of a dendron, perfect dendrimer, dendritic polyglycerol, and hyperbranched polymer. Modified from nanopartica 2015, Paulus 2014, Quadir and Haag 2012

Dendrons and dendrimers are organised very symmetrically and possess a high monodispersity. A limiting factor for their application is the time-consuming nature of their preparation in a multi-step synthesis. Hyperbranched polymers are irregular, randomly

branched constructions. In contrast to dendrimers, hyperbranched polymers show no distinguishable interior and periphery, which makes them unsuitable for complex polymer architecture. They exhibit a broad polydispersity but can be prepared in a one-step reaction (Frey and Haag 2002; Tomalia et al. 2012). For PG as precursor of dPGS in this study the DB is 60%, which is lower than the 100% of perfectly branched glycerol dendrimers (Türk et al. 2004). However, PG is expected to possess physicochemical properties similar to those of perfect dendrimers, such as supramolecular void spaces that non-covalently harbour drugs or complex core-shell architectures. Additionally, PGs can be synthesised in a commercially applicable one-step process, which is based on anionic, ring-opening multibranching polymerisation (Frey and Haag 2002).

Furthermore, in contrast to their linear counterparts the globular structure of these branched polymers allows an exceptionally high number of functional groups. PG for example possesses terminal hydroxyl groups which allow simple post-modification in a second step synthesis (Calderón et al. 2010). The large numbers of functional groups render dendritic polymers suitable as nanocarriers and enable multivalent interaction with biological receptors or other chemical entities. As multivalency is the most predominant phenomenon in biological systems and is considerably stronger than a corresponding number of monovalent ligands binding individually to a multivalent receptor, PG might offer, together with its nanoscale size, a number of structural advantages for modulating biological functions (Calderón et al. 2010; Fasting et al. 2012; Quadir and Haag 2012).

Due to the aliphatic polyether backbone, PG is believed to be as highly biocompatible as the benchmark polymer poly(ethylene)glycol (PEG; Calderón et al. 2010; Frey and Haag 2002). Initial toxicological examinations of dPGS were also promising as it exhibited no cytotoxic effects *in vitro* (Khandare et al. 2010). Similarly, in a mouse dermatitis model (Dernedde et al. 2010), in a rat diagnostic imaging model (Licha et al. 2011), and in a mouse asthma model (Biffi et al. 2013) fluorescently labelled dPGS did not lead to any obvious, acute adverse effects. However, comprehensive studies of the *in vivo* toxicology of dPGS were still missing and the kinetics, including any potential unintended accumulation that may lead to long-term effects, had not been investigated. Both are essential information before a compound can be considered as a diagnostic or therapeutic agent (Almeida et al. 2011).

2.3.3   Polymer Characterisation - dPGS and dPGS amine Preparation

The synthesis of dPGS was not part of this study and is therefore described only briefly. In preliminary work, the Haag group used PG (DB 60%) as core polymer with SO_3 pyridine complex in a concentration equimolar to the OH groups as sulfation reagent to obtain

dPGS. The resulting dPGS possessed sulfate ester groups with a DS of 85%, leading to a negatively charged, polyanionic surface (Türk et al. 2004).

The dPGS with additional amino functions (dPGS amine or radioactive dPG^{35}S amine) was prepared by first synthesising dendritic polyglycerol containing 9.5% phthalimide functionalities (Gröger et al. 2013). For radioactive dPG^{35}S amine the sulfation agent ^{35}SO$_3$ pyridine complex was obtained from pyridine and ^{35}S-chlorosulfonic acid that had been previously synthesised. Sulfation was carried out as described above. The free amino functions were obtained in a Gabriel-type hydride mediated hydrolysis of the phthalimide. A polymer with a DS of 85%, as determined by elemental analysis, and a number-average molecular weight of 10.05 kDa and 9.5% amino groups, corresponding to 4-5 functionalities on average per polymer, was obtained. The resulting dPGS amine is only an intermediate product. The amino functionalities were generated to allow for straightforward conjugation of drugs or dyes and are derivatised during conjugation (Dernedde et al. 2010; Gröger et al. 2013; Licha et al. 2011). Dynamic light scattering (DLS) in phosphate buffered saline (PBS) at pH 7.4 revealed a hydrodynamic diameter (HD) of 5.7 ± 1.5 nm, a size previously found for dPGS with a comparable core size and DS bearing no amino functionalities (Weinhart et al. 2011a; Weinhart et al. 2011b). Under the applied conditions, partial aggregation was found using DLS, probably due to charge-based intermolecular interactions of sulfate and amino functions. This finding was further supported by a zeta potential of -31 ± 6 mV, which was more negative than a typical zeta potential of non-aggregated dPGS without amino groups of comparable size, DB and DS (Weinhart et al. 2011b). The radiolabelled dPG^{35}S amine and dPGS amine were subsequently used to establish the biodistribution studies of this study.

2.4   Biodistribution of Nanoparticles

How NPs distribute throughout an organism is influenced by a variety of factors, including the complex particle properties, the environment of the NP, and the route of administration (Owens Iii and Peppas 2006). The following factors are known to affect the distribution of NPs. However, to date the precise interrelations of particle properties and distribution often remain unclear and the literature lacks consistency, though general trends are discernible (Owens Iii and Peppas 2006).

2.4.1   Protein Corona

When the NP enters the bloodstream, its surface is immediately covered by a layer of biomolecules (mostly proteins), called "biomolecular corona" or "protein corona" (Lundqvist

et al. 2011). The corona not only increases the HD (Almeida et al. 2011) it also gives the NP a biological identity that differs from its synthetic identity (Walkey and Chan 2012). The determinants which control the residency of biomolecules on the particle surface are very complex, and describing them with association/dissociation rate constants may not be completely accurate (Monopoli et al. 2012). Size (curvature), shape and physicochemical surface characteristics such as hydrophobicity play a role (Cedervall et al. 2007a; Lundqvist et al. 2011; Schleh et al. 2012) as well as the ratio of NP surface area to protein concentration (Cedervall et al. 2007b; Lundqvist et al. 2004).

Besides the composition and organisation of the biomolecules on the NP surface, the exchange rates of the biomolecules are crucial determinants for the biological identity. While the outer, loosely associated layer of biomolecules undergoes exchange within seconds, an inner layer, the hard corona, remains associated with the surface of the NP long enough to be biologically significant (Cedervall et al. 2007a; Monopoli et al. 2012). It is assumed that the hard corona is highly stable and is only partially replaced when entering a new environment. In contrast to biomaterials, which remain stationary at the site of implantation, the biomolecular corona may therefore depend on all environments it has passed through and not only on the current environment (Lundqvist et al. 2011; Monopoli et al. 2012). Moreover, biomolecules in the corona such as apolipoproteins, found in the corona of very different NPs (Cedervall et al. 2007b; Lundqvist et al. 2008; Staufenbiel et al. 2014; Walkey and Chan 2012), may be relevant to the transport process itself and may also influence the crossing of biological barriers, e.g. via scavenger receptors (Monopoli et al. 2012; Schleh et al. 2012; Staufenbiel et al. 2014).

2.4.2 Tissue Perfusion

The perfusion of an organ and the permeability of the endothelium also affect the magnitude of the distribution of the NP into the different tissues. Tissues that are highly perfused and have a fenestrated endothelium, such as the kidney, or a discontinuous endothelium, as in the liver, spleen, and bone marrow, are more likely to be entered by NPs (Li et al. 2010; Li and Huang 2008).

2.4.3 Clearance

In the kidney, globular NPs with an HD of less than 6 nm are typically filtered and excreted via the urine, while particles above 8 nm will not be filtered by the glomerular capillary endothelium (Soo Choi et al. 2007). In the intermediate 6-8 nm size range, filtration is also affected by the charge of the particle (Choi and Frangioni 2010; Longmire et al. 2008). The charge selectivity is attributed to the negatively charged glomerular basal membrane

(Longmire et al. 2008). The molecular weight threshold for renal filtration of NPs differs considerable in the literature. For polymers, a threshold of 30-50 kDa is reported (Fox et al. 2009; Lee et al. 2005). This threshold is higher for flexible and linear particles than for rigid and globular structures (Choi and Frangioni 2010). In the case of polymers this means the DB determines the filtration above a certain molecular weight. Highly branched dendrimers, for example, were found to be excreted nine times less compared to less compact polymers with a similar molecular weight (Fox et al. 2009; Lee et al. 2005). In the proximal tubular epithelial cells some NPs may be reabsorbed, thus partially or fully negating the effect of glomerular filtration. Studies concerning this reabsorption are, however, limited (Longmire et al. 2008; Rennke et al. 1978).

If not cleared by the kidney, NPs can either undergo biodegradation to clearable components and be excreted through the liver into the bile and into the faeces or they are sequestered by cells of the Reticuloendothelial System (RES) or more specifically the Mononuclear Phagocyte System (MPS); subsequently referred to as MPS. With respect to biliary excretion, an inverse correlation of particle size and contribution of hepatocytes to total hepatic uptake was found for silica NPs, gold NPs, and PEGylated quantum dots (Cho et al. 2009; Choi et al. 2009; Hirn et al. 2011; Roerdink et al. 1984). The accessibility of hepatocytes reflects the size of the fenestrations in the hepatic sinusoidal endothelium. On average the diameter of the fenestrae is around 140 nm in mice (Moghimi et al. 2001; Wisse et al. 2008). Due to slow hepatic processing and biliary excretion, particles in the liver often show a prolonged retention (Longmire et al. 2008), emphasising the importance of the cellular localisation in this highly exposed organ.

2.4.4   Uptake in Mononuclear Phagocyte System

Particle with an HD above 200 nm were found to typically experience rapid uptake by, for example, Kupffer cells of the liver and other cells of the MPS (Owens Iii and Peppas 2006). Internalisation is enhanced by a process called opsonisation. Specific proteins, the opsonins, mark the NP for recognition by MPS cells. The most common opsonins are immunoglobulins and complement proteins such as C3 after cleavage (Owens Iii and Peppas 2006). Other well-known opsonins are fibrinogen gamma (γ) and beta (β) chains (Staufenbiel et al. 2014). The affinity of an opsonin is specific to the NP properties. Furthermore, macrophages and monocytes show heterogeneity in phenotype and physiological properties (e.g. phagocytosis). They may, therefore, respond to different opsonins, which in return may contribute to the difference in responsiveness of the MPS organs (Moghimi et al. 2001; Moghimi and Patel 1988). NPs that are not degraded by the macrophages are typically sequestered in MPS organs and consequently might lead to long-term, negative side effects (Owens Iii and Peppas 2006).

2.4.5   Avoiding the Mononuclear Phagocyte System

To date there are no absolute methods available to effectively block the opsonisation of NPs (Owens Iii and Peppas 2006). However, to hinder this non-specific protein binding and prevent immediate clearance from the blood stream, scientists began to "stealth" the surface of the NPs. This means the surface is modified to decrease the interaction with plasma proteins and avoid opsonisation. The surface modifications most often used are the polymer PEG and PEG-containing copolymers (Owens Iii and Peppas 2006). PEG is hydrophilic and neutrally charged (Owens Iii and Peppas 2006).

In general, the protein absorption is higher for hydrophobic than for hydrophilic NP and for charged than for neutral NP (Owens Iii and Peppas 2006). For dendrimers this means, the interaction with the physiological environment, apart from size, is mainly controlled by the charge of their terminal groups (Choi and Frangioni 2010).

2.5   Theoretical Biodistribution of dPGS amine

The partially aminated dPGS with a HD of 5.7 nm ± 1.5 nm, surveyed in this work, possesses a size slightly below the renal threshold. According to literature, those particles are rapidly cleared by the kidney (Soo Choi et al. 2007), leading to a short blood half-life and low uptake in MPS organs. However, the close proximity to the renal threshold together with the negative charge resulting from a DS of 85% and the rigidity due to a DB of 60% might also bear the risk of restricted filtration (Longmire et al. 2008). Longer circulating dPGS could be excreted via the hepatobiliary pathway, presumably up to a size of 140 nm (Moghimi et al. 2012), but preferentially in the range of 10-20 nm (Soo Choi et al. 2007).

The charged surface of dPGS amine might also enhance opsonisation. In previous *in vitro* studies dPGS was shown to bind complement factor C3, which is a common opsonin upon cleavage (Dernedde et al. 2010). Furthermore, the additional amine groups were found to lead to partial aggregation of dPGS amine in PBS under a physiological pH of 7.4 by DLS (Gröger et al. 2013). Aggregation would enhance uptake in MPS cells, resulting in a rapid clearance from the blood-stream. In contrast to aggregation, the amino groups may also lead to a back-folding of the sulfate groups and the lower repulsion might also decrease the HD of the individual dPGS amine compared to dPGS with the same molecular size and DB (Weinhart et al. 2011b).

The following section will provide an overview of possible detection methods of NPs *in vivo,* which could be used to assess the dPGS biodistribution.

2.6 Detection of Nanoparticles in Tissue *in vivo*

Due to their small size, the detection of NPs entails some special challenges. Besides microscopic techniques, radioisotopes or analytical techniques such as Raman spectroscopy are commonly used for NP detection. None of these techniques alone allows an analysis that includes NP localisation with an assignment to cells (Vanhecke et al. 2014), quantification, and simultaneous histopathological evaluation of surrounding tissue. Yet, these are the parameters required to assess the therapeutic efficacy as well as dose-dependent safety and toxicity issues. For a better understanding of this challenge, the strength and limitations of the most commonly used techniques are discussed in the next section.

2.6.1 Microscope-Based Approaches

2.6.1.1 Light Microscopy

The resolution of light microscopy, the most often used technique in routine standard histopathological diagnostic, is restricted by the diffraction limit of light, which depends on the wavelength of the light and the optical resolution of the light microscope (Abbe 1873), resulting in a lateral resolution capacity above 200 nm and an axial resolution capacity of about 900 nm (Smith 2001; Vanhecke et al. 2014). Given the dimension of NPs, light microscopy is thus insufficient to describe NPs individually. NPs can only be visualised directly when aggregated to clusters that exceed 200 nm. Furthermore NPs, or rather their aggregates, must have sufficient contrast on their own to stand out against the biological context during histological standard procedures or be stainable with (histological) dyes. Aggregates of the soft NP dPGS for example can be visualised by Alcian blue, a histological stain for negatively charged sulfate groups (Gröger et al. 2013; Scott 1972). Nevertheless, light microscopy remains a valuable standard technique to evaluate NP-associated changes in a cellular phenotype (Ostrowski et al. 2015; Vanhecke et al. 2014).

2.6.1.2 Fluorescence-Based Techniques

2.6.1.2.1 Fluorescent (Wide Field) Microscopy

Fluorescent (wide field) microscopy was first applied to detect NPs with intrinsic fluorescent properties such as quantum dots. Conjugation of NP with fluorescent dyes enabled the detection of a wider range of NPs in cells or tissues (Elsaesser et al. 2010). The inability to visualise non-fluorescent structures of a cell in parallel impedes histopathological evaluation. Selective counterstaining, e.g. by blue fluorescent dye 4´,6z-diamidino-2-phenylindole (DAPI), can provide individual information such as the size and shape of the nucleus but is insufficient for a complete histological assessment (Ostrowski et al. 2015). Further drawbacks are the time-dependent photobleaching, intracellular quenching or

uncoupling of fluorescent dyes, which have to be considered when evaluating NP biodistribution (Ostrowski et al. 2015; Vanhecke et al. 2014). Moreover, labelling of NPs with a fluorophore changes the chemical structure. The dPGS particle, as used for example in this study, possesses a molecular weight of 10,050 Da (Gröger et al. 2013), whereas the fluorescent dye indocarbocyanine 3 has a molecular weight of 767 Da. The increase in size and/or the changes in the chemical structure are likely to result in altered physicochemical properties, thus quite possibly leading to a different interaction with biomolecules, cells and tissues (Marquis et al. 2009; Müller et al. 1997).

2.6.1.2.2 Confocal Microscopy

Improved optical resolution particularly in the axial dimension, compared to traditional (wide field) fluorescence microscopy as described above, was achieved by the development of confocal microscopy, where a spatial pinhole system eliminates the out-of-focus light. Two confocal microscopic techniques are generally in use: the confocal laser scanning microscopy (LSM) and the spinning disc confocal system (Vanhecke et al. 2014). LSM enhances the resolution in the axial-dimension to 450-550 nm, in the lateral dimension to approximately 150 nm (Hell 2007; Smith 2001). Enhanced 4 Pi microscopy even enables a resolution in the axial dimension of 80-150 nm to be obtained (Hell 2007). Furthermore, LSM allows for optical sectioning and 3D reconstruction of a tissue. A disadvantage is the longer exposure time needed. The spinning disc system suffers from a poorer axial resolution but is qualified for fast dynamic processes (Vanhecke et al. 2014).

2.6.1.2.3 Super-Resolution Light Microscopy

An enhanced resolution, relying on an "on- and off-switching" of fluorescent states of objects that are closer together than the diffraction limit, is provided by several super-resolution techniques such as stimulated emission depletion microscopy (STED), or photoactivated localisation microscopy (PALM) and stochastic optical reconstruction microscopy (STORM; Hell 2009). Such techniques can enable axial resolution capacities of approximately 30 nm 50 nm respectively and a lateral resolution capacity of approximately 20 nm, thus permitting the individual detection of larger NPs. Furthermore, super-resolution microscopy allows for 3D and live imaging of subcellular structures (Bates et al. 2008). However, to date super-resolution light microscopy requires relatively long acquisition times at high resolutions (Zhuang 2009) and is not yet a commonly available diagnostic tool (Elsaesser et al. 2010).

An advantage of fluorescent microscopy in general is the possibility of real-time imaging. However, like fluorescent (wide field) microscopy, confocal and super-resolution light microscopy generally require labelling and do not allow for a histopathological evaluation in parallel.

2.6.1.3 Electron Microscopy

With a resolution power down to a few angstroms, electron microscopy easily overcomes the resolution limits of light microscopy. Because no specific labelling is required, scanning electron microscopy and especially transmission electron microscopy (TEM) provide a pristine visualisation of NPs both *in vitro* and *in vivo* (Elsaesser et al. 2010; Vanhecke et al. 2014). Furthermore, detailed information on subcellular structures as well as potential pathological changes can be provided (Terje et al. 2006). However, electron microscopy is restricted to electron-dense, high atomic number material that has a sufficient contrast in biological material (Elsaesser et al. 2010; Marquis et al. 2009; Vanhecke et al. 2014). Another limitation is the requirement for ultra-thin sectioning, as electrons do not penetrate a sample beyond a depth of a few 100 nm. Consequently only low volumes can be processed and ultrathin sectioning is prone to artefacts (Elsaesser et al. 2010; Vanhecke et al. 2014). One possibility of overcoming volume restrictions is 3D reconstruction, which uses serial 2D sectioning or tomography. Such 3D construction requires intensive computing power as well as time to reconstruct a certain volume (Elsaesser et al. 2010).

Scanning transmission electron microscopy (STEM) is an enhancement of TEM and SEM is the. One of its principal advantages over TEM is its ability to use other signals, with examples including, elemental analysis techniques such as energy dispersive (x-ray) spectroscopy (EDX) or electron energy loss spectroscopy (EELS), which can be spatially correlated to the TEM image (Elsaesser et al. 2010). Such an elemental analysis can be used for quantification, or for distinguishing NPs from artefacts (e.g. metal precipitates from stains; Hall et al. 2007). EELS is also capable of mapping the chemical composition of biological compounds such as proteins and water by detecting the elements nitrogen, sulfur, phosphorus carbon, and oxygen (Aronova and Leapman 2012). For biological components the dose-limited resolution is about 50 nm (Aronova and Leapman 2012), for solvated polymers about 10 nm (Yakovlev and Libera 2008) before suffering damage. Detection of NPs composed of the elements listed above should theoretically also be possible in confined sites of interest, where the elemental fingerprint is distinguishable from those of the biological components. At present such techniques are still both time-consuming and technically demanding

2.6.1.4 Quantification of Microscope-Based Approaches

In general, microscope-based approaches usually produce single images of a very small pane, not necessarily representative of the entire sample, thus rendering quantification difficult (Elsaesser et al. 2010). Stereology is the statistical extrapolation of the amount of NP from representative plane sections to achieve quantification, using the fundamental principles of geometry and statistics. To obtain unbiased information, systematic sampling

is applied following statistical principles that are the same as those of survey sampling (Elsaesser et al. 2010; Vanhecke et al. 2014).

2.6.2 Radioactivity Bases Approaches

The incorporation of a radioisotope into a compound is a very sensitive way of detecting and quantifying NPs (Christensen et al. 2014; Elsaesser et al. 2010; Kreyling et al. 2009). Compared to dye tags, the NP properties are unaltered when the naturally occurring atoms within a macromolecule are exchanged for their radioactive isotope (Christensen et al. 2014). The most commonly used radioisotopes are carbon (^{14}C) or tritium (^{3}H), because they can be incorporated into nearly any organic material, and sulfur (^{35}S) or iodine (^{125}I; Baker 1989).

The radiolabel has to be localised in a stable and defined position given potential metabolism in the biological environment. The best stability is reached when the radioisotope is well integrated. This depends on the composition of the NP as well as on the nature of the radioisotope. Depending on the study design, the half-life of the radioisotope and the emitted energy also have to be taken into account. In contrast to a high energy radioisotope, such as ^{125}I, low energy isotopes ^{3}H and ^{14}C produce an image in the detection layer close to their site of origin, resulting in a high resolution (Baker 1989; Solon 2015). It should be noted that the handling of radioisotopes is restricted by safety precautions and governmental regulations including the safe disposal of blood and tissues (Elsaesser et al. 2010).

2.6.2.1 Liquid Scintillation Counting

Liquid Scintillation Counting (LSC) is a highly sensitive method to quantify low energy, mostly from beta-emitting and alpha-emitting radioisotopes. The sample containing the radioactive material is mixed with a liquid, chemical cocktail that is able to convert the kinetic energy of nuclear emissions into light energy. The light is detected using a photomultiplier tube (PMT). The number of flashes of light is recorded in a preselected time period expressed in counts per minute (cpm; L'Annunziata 2012). The pulse height of the PMT signals is proportional to the nuclear decay energy, which allows a sorting of isotopes on the basis of their particular energy range (L'Annunziata 2012). Most counters have two photomultiplier tubes connected in a coincidence circuit. The main advantage of this reconciliation is the reduction of background rates, due to effects such as line noises or luminescence (Horrocks 1974). In addition, the two PMTs permit coincidence pulse summation to detect low energy radionuclides such as ^{3}H (L'Annunziata 2012).

Besides false background counts (cosmic radiation/line noises or luminescence), the signal recovery can also be reduced due to factors such as chemical or colour quench. This has to be taken into account during measurement by performing a quench correction (L'Annunziata 2012; Scales 1963). It should be noted that LSC has a destructive effect on the sample and does not provide information on cellular NP localisation or tissue alterations.

2.6.2.2 Autoradiography

Autoradiography is the term for different techniques which use x-ray films, phosphor imaging plates and photo-nuclear emulsions to produce an image that reflects the pattern of the radioactive decay.

2.6.2.2.1 Whole Body Autoradiography

Whole body autoradiography (WBA) and whole organ autoradiography (WOA) have been used for decades to obtain autoradiographs of the tissue distribution of a radiolabelled drug. This involves exposing tissue sections to x-ray film. The resolution depends on many factors such as the energy of the radioisotope, exposure and developing time, the distance between sample and recording layer, as well as the film type (i.e., the silver-halide grain size of the emulsion layer and its thickness; Kerkápoly et al. 2005). Accordingly, the selection of the film should be complementary to the energy of the radioisotope, but the relative importance of a quick result can also affect the choice of the film. The grain size of the silver halide grains of 0.2-0.4 µm in diameter leads to a high spatial resolution (Baker 1989; Cabello and Wells 2010). The images obtained can provide powerful information about the distribution of a drug or NP. However, autoradiography does not allow for any information on the cellular level (Baker 1989; Solon 2015). Furthermore, due to the inherent non-linearity of films, attempts to quantify the radioactivity were of limited success; only semi-quantitative data could be obtained (Solon et al. 2010).

2.6.2.2.2 Quantitative Whole Body Autoradioluminography

Body sections or organ sections are exposed to phosphor imaging plates along with radioactive calibration standards. The imaging plates are scanned using a phosphor imager. This is able to achieve a digital grey-scaled image with a resolution (pixel size) of 25 µm and more (CR-35 HD, Dürr, Germany; Typhoon FLA 7000 Laser Scanner, GE Healthcare Life Sciences, USA; Imaging Plate Scanner Micron25, Ditabis, Germany). Compared to analogous autoradiography, autoradioluminography offers less resolution power but allows for shorter exposure times (Cabello and Wells 2010; Solon and Kraus 2001). Furthermore, phosphor-imaging technology also enables quantification (Solon 2015). Quantitative evaluation is performed by image densitometry using image analysis software and a calibration standard. The concentrations of radioactivity are determined in Bq/g (Solon 2015).

Today quantitative WBA (QWBA) has replaced WBA and is the standard method for conducting tissue distribution studies as part of a preclinical drug approval process. Quantitative autoradioluminography has also already been applied for the detection of NPs (Christensen et al. 2014; Solon 2015). Its main advantage is the low effort in sample processing (Solon 2015). Unfortunately, similar to WBA, QWBA does not provide data at the microscopic level.

2.6.2.2.3 Light Microscopic Autoradiography or Microautoradiography

Light microscopic autoradiography (LMA) has previously been used for drug approval studies or for the localisation of specific nucleic acid sequences, such as chromosomes during *in-situ* hybridisation. In this study, the LMA technique was set up for detection of the soft NP dPGS at the cellular level.

LMA provides a spatial localisation of radiolabelled drugs at tissue and cellular level in histological samples. There are many variations of this method in literature (Stumpf 2013). One is the method of Appelton and Stumpf, where cryo-sectioned tissue samples of 4-10 µm are thaw-mounted onto dry, pre-coated glass slides. Another approach is to mount formalin-fixed, paraffin-embedded (FFPE) tissue sections on dry glass slides followed by immersion of the slides in aqueous emulsion (Baker 1989). Both procedures are conducted under safe light conditions. The exposure times depend on the specific activity of the compound, its concentration in the tissue, and finally the desired resolution. They can last from days to weeks and have to be determined empirically (Stumpf 2005). Following exposure of the emulsion to the section, the slides are developed in a similar way to photographic films (Baker 1989; Solon 2015). Subsequently the slides are stained following standard histological staining procedures. Staining may also include immunohistochemistry (IHC; Solon 2015).

The grain size of the emulsion (e.g. 0.2 µm Ilford K5D) and the resolving power of the microscope determine the optical resolution. Due to the option of adapting exposure times for compounds with low specific activity or for weak tissue concentrations, the information on the cellular localisation can be obtained with high sensitivity (Stumpf 2005). Moreover, the region of the NP localisation can be evaluated simultaneously by histopathological examination (Stumpf 2013). A major drawback is the time-consuming acquisition. Furthermore, the procedure requires a high level of skill on the part of the analyst, since artefacts, such as light leaks, reticulation of emulsion, or crystalline deposits from the developing process, can accrue easily (Gahan 1972; Solon 2015). Quantification was carried out by graticule counting or photometry systems (Baker 1989) but generally is laborious and problematic, among other reasons because of the lack of internal standardisation facilities.

2.6.2.2.4 Electron Microscopic Autoradiography

Autoradiography has also been used along with electron microscopy to examine the distribution at the subcellular level. For this purpose, emulsions with lower grain size, (e.g. 0.11 µm Illord L4) are applied. Within a study of neurotrophin transport, it was shown that grain counting can be used to provide a quantitative characterisation of the observed distribution (Solon 2015).

2.6.3 Analytical Techniques

2.6.3.1 Raman Spectroscopy

Raman spectroscopy relies on inelastic scattering of light of a single wavelength, usually from a laser as a monochromatic source. The light interacts with molecular vibrations, resulting in the energy of the laser photons being shifted up or down. The received Raman spectrum provides information on the vibrational modes of the molecules that are specific to the chemical bonds and symmetry of the molecules. The advantage of Raman spectroscopy lies in the high chemical specificity. Based on biochemical spectra information, it is then possible to determine the quantitative composition of a sample as well as information on molecular changes in cells, such as those due to pathological processes (Keating and Byrne 2013; Kong et al. 2015). Furthermore, Raman spectroscopy is a non-destructive technique and information can be obtained without the need for staining or labelling (Kong et al. 2015).

Raman spectrometers can also be efficiently coupled with optical microscopes because the wavelengths of the excitation lasers lie in the visible and near-infrared region. The spatial resolution of Raman microspectrometers is about 300-500 nm. It is therefore not suitable for single NP visualisation. However, the localisation of NPs can be assigned to cells and, moreover, molecular changes in these cells can be determined (Kong et al. 2015).

A major drawback of Raman spectroscopy in general is the low signal intensity of the inelastic light scattering compared to the elastic scattering or fluorescence emission. Long acquisition times are needed to prevent an insufficient signal-to-noise ratio or spatial under-sampling. To resolve this limitation new strategies are currently under development. These include imaging modalities based on non-linear Raman scattering, multimodal integration and selective Raman microscopy to name just a few (Kong et al. 2015). Because Raman spectroscopy also allows detection and quantification of low atomic number material, such as organic NPs, without labelling, it is an interesting tool, but one for the future. To date, there is still a long way to go until it will be suitable for implementation as a standard research method or in drug approval studies.

3   AIMS AND HYPOTHESIS OF THIS STUDY

The NP dPGS is a promising therapeutic and imaging tool for inflammatory diseases. Since the development of dPGS for biomedicine is a rather new area, no data has been available either on the biodistribution or toxicity in target organs of healthy animals or on a detection technique in which the structure of the polymer remains unaffected. Both are mandatory for a safety assessment and subsequent drug approval. The work presented here intended to analyse the kinetics and determine first insights into the general toxicity of dPGS based on the pathomorphological evaluation of tissues and cells following its i.v. or s.c. application. To facilitate a detailed biodistribution study, including the cellular localisation and a histopathological evaluation of potential alterations, the LMA technique was established for dPGS detection. The following hypothesis and aims were postulated for this present study.

3.1   Hypothesis: The NP dPGS is a suitable candidate for biomedical applications

The NP dPGS was designed on the basis of the highly biocompatible PG and *in vitro* studies of dPGS have also shown a high biocompatibility of the sulfated dPG (Khandare et al. 2010). Furthermore, during *in vivo* studies with fluorescently labelled dPGS on animal models for specific diseases, no obvious acute clinical signs or gross lesions in the animals were observed until 3 and 24 hours respectively after the application of therapeutic doses (Biffi et al. 2013; Gröger et al. 2013; Licha et al. 2011). However, systematic survey data on the biodistribution of dPGS and its possible toxicological impact were still missing. **The overall hypothesis of this study was that dPGS is a suitable candidate for biomedical applications**. To test this hypothesis, both a kinetic as well as systematic gross and histopathological analysis were necessary, as is required for the safety assessment in preclinical drug approval according to the guidance document M3(R2) of the ICH (ICH 2009) in the absence of other, specific regulations for NPs.

3.1.1   Aim No. 1: Establish a technique that would make it possible to determine the unadulterated cellular localisation of the dPGS NPs and to assess corresponding cellular alterations

The detection of soft NPs such as dPGS is especially challenging. Commonly, fluorescent dyes are used to assess their tissue and cellular localisation. Fluorescent tagging, however, has been shown to alter the surface characteristics as well as the protein pattern absorbed to the surface of the particle (Müller et al. 1997). In a previous study using dPGS conjugated with new-indocyanine-green in a rat arthritis model, the fluorescently conjugated dPGS was found not only at the sites of inflammation but also in the liver of the animals. In the same study additionally administered dPGS conjugated with indocarbocyanine (ICC) was assigned to the Kupffer cells of the liver (Licha et al. 2011). Another study using ICC but a different

linker motive for conjugation also found dPGS in the Kupffer cells and additionally in the spleen (Gröger et al. 2013). Uptake into cells of the MPS is a rather common phenomenon for "unstealthed", charged NPs and those exceeding a certain size (Owens Iii and Peppas 2006). The first question concerned whether unconjugated dPGS exhibits the same behaviour and the second whether the presence of dPGS leads to histopathologically detectable alterations in these cells. The **first aim** of this present study was therefore to find a technique that would make it possible to determine the unadulterated cellular localisation of dPGS NPs and to assess the corresponding cellular alterations. Furthermore, such a technique needed to be generally applicable and not only feasible for well equipped, highly specialised laboratories that are able to apply techniques such as Raman microspectroscopy.

Radioisotopes have already been used to label other NPs (Kainthan and Brooks 2007). Depending on the isotope used, the chemical structure and characteristics remain the same. Because the dPGS macromolecule possesses an outer shell with a large number of sulfate groups (Türk et al. 2004), dPGS was synthesised in advance of this study with the radioisotope ^{35}S (Gröger et al. 2013). The radiation of ^{35}S can be traced under a light microscope by a technique called LMA. This technique has already been used for some time in pharmaceutical research and for *in situ* hybridisation (Baker 1989; Solon 2015) but not for NP detection. If also applicable to radioactively labelled dPGS, it would perform the task of detecting dPGS at its unadulterated cellular localisation and would facilitate simultaneous histopathological evaluation.

3.1.2   Aim No. 2: Perform a kinetic study that reveals the time-dependent tissue concentrations, cellular localisation, and excretion as well as the terminal blood half-life of radioactive dPG^{35}S amine in the major organs after i.v. or s.c. application to mice

The uptake of fluorescently labelled dPGS raised concerns regarding the potential accumulation of dPGS in the MPS. For a therapeutic and diagnostic imaging tool, accumulation is undesirable because of potential long-term effects or its interference with other diagnostic tools (Choi and Frangioni 2010). In contrast, clearance within a reasonable period of time would prevent such effects. During kinetic studies, the interaction of a compound with the organism is analysed, among other characteristics, by its distribution and excretion. The tissue concentration obtained for a compound and the duration of its residency are important for the interpretation of its toxicology and pharmacology (ICH 1994). The **second aim,** therefore, was to perform a kinetic study that would reveal the distribution with the time-dependent tissue concentrations to determine the potential sites of accumulation, the cellular localisation as well as the route of excretion and the terminal blood

half-life as a parameter for retention in the mouse body. Besides i.v. injection, the s.c. mode of application was of interest because of its applicability to self-medication by outpatients.

3.1.3 Aim No. 3: Provide first evidence towards the potential acute and subacute general toxicity of dPGS on the basis of macropathology and histopathology.

Previous studies using dPGS were either performed in specific animal models such as the rat arthritis model and/or investigated with fluorescently labelled dPGS. The results of those studies, though promising due to the absence of obvious adverse effects, lacked a systematic evaluation of safety issues. Furthermore, those studies failed to provide insight into the potential pathomorphological changes on tissue and cellular level which are based on an unadulterated distribution of dPGS administered to healthy animals, as commonly performed in preclinical toxicity studies. The **third aim,** therefore, was to provide first evidence on the potential acute and subacute general toxicity of dPGS on the basis of a macropathological and histopathological evaluation in healthy mice.

4   RESEARCH PUBLICATIONS IN SCIENTIFIC JOURNALS

4.1   Tissue and cellular localization of nanoparticles using ^{35}S labeling and light microscopic autoradiography

Authors: Holzhausen C, Gröger D, Mundhenk L, Welker P, Haag R, Gruber AD

Year: 2013

Journal: Nanomedicine, Nanotechnology, Nanobiology 9(4), 465-468

Declaration of own portion of work in the research publication:

Contribution of C. Holzhausen: Design, preparation, completion and evaluation of all experiments presented and subsequent creation of the entire manuscript except for the synthesis and characterisation of dPGS amine and dPG^{35}S amine.

Contributions of other authors: All authors participated in the development of the study design, the evaluation of experimental results and the review of the manuscript. The synthesis and characterisation of dPGS amine and dPG^{35}S amine were performed by D. Gröger.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (State Office of Health and Social Affairs, Berlin, approval ID G 0028/10) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).
http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

Tissue and cellular localization of nanoparticles using ^{35}S labeling and light microscopic autoradiography

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For legal reasons the journal article is not published here,
but can be purchased online.

4.2 Biodistribution, cellular localization, and in vivo tolerability of ^{35}S-labeled antiinflammatory dendritic polyglycerol sulfate amine

Authors: Holzhausen C, Gröger D, Mundhenk L, Donat C, Schnorr J, Haag R, Gruber AD

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Declaration of own portion of work in the research publication:

Contributions of C. Holzhausen: Design, preparation, completion and evaluation of all experiments presented and subsequent creation of the entire manuscript except for the synthesis and characterization of dPGS amine and dPG^{35}S amine.

Contributions of all authors: All authors participated in the development of the study design, the evaluation of experimental results and the review of the manuscript. The synthesis and characterisation of dPG^{35}S amine and dPGS amine were performed by D. Gröger. Furthermore, D. Gröger made a considerable contribution to the liquid scintillation counting measurement.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the local governmental authorities (State Office of Health and Social Affairs, Berlin, approval ID G 0028/10) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).
http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

Biodistribution, cellular localization, and in vivo tolerability of 35S-labeled antiinflammatory dendritic polyglycerol sulfate amine

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5   CONCLUDING DISCUSSION

With its anti-inflammatory characteristics, as demonstrated *in vitro* and also *in vivo* in preliminary selectin binding assays, in a dermatitis mouse model as well as in an arthritis rat model, dPGS is a potential therapeutic and diagnostic agent for inflammatory conditions. However, while the LD50 of dPGS had been established prior to this study (unpublished work), no data concerning the kinetics (ADME) and the toxicopathology of dPGS were available. Yet these data are crucial in order to understand which organs may be affected by toxicity and, subsequently, to review the safety and efficacy of a candidate drug. For the purposes a toxicological assessment, it is also necessary to evaluate alterations in the morphology of tissues and cells. For such an evaluation it is especially valuable to conduct a cellular localisation of the administered compound in parallel to a morphological analysis. Due to the fact that the assessment of dendritic polymers *in vivo* is still a newly emerging area, labelling techniques for quantification and visualisation down to the cellular level were required.

The most commonly applied as well as economically justifiable technique for the visualisation of NPs at the cellular level is fluorescent labelling, but it substantially changes the chemical structure of a NP and has a great influence on its physical and chemical characteristics. In addition, it does not allow for an evaluation of the cellular morphology. The method of choice for kinetic studies is radiolabelling. It is a suitable technique for preventing modification of the properties of the NP and supports the visualisation and quantification of the tissue distribution. Its applicability, however, might rely upon the ability to integrate the isotope into the chemical structure of the NP. Furthermore, commonly applied techniques such as QWOA, WOA or LSC are not suitable for localising NPs in cells or for detecting cellular alterations.

A method to detect radioactively labelled NPs on cellular level would cover both hitherto unsolved aspects: the assessment of an unadulterated biodistribution down to the cellular level on the one hand and the evaluation of cellular alterations on the other.

Such a method was implemented in this study and the tolerability of partially aminated dPGS (dPGS amine) NPs was evaluated on the basis of kinetic analysis and pathomorphological evaluation *in vivo*. The following sections discuss the hypothesis and aims of this study as well as the potential of dPGS for clinical translation.

5.1 Hypothesis: The NP dPGS is a suitable candidate for biomedical applications

5.1.1 Aim No. 1: Establish a technique that would make it possible to determine the unadulterated cellular localisation of the dPGS NPs and to assess corresponding cellular alterations

In former studies fluorescently tagged dPGS was used to evaluate its efficacy in inflammatory conditions *in vivo*. However, fluorescent labelling may be unreliable and may not fulfil the aims as postulated above.

5.1.1.1 Fluorescent Dyes Used for dPGS Detection *in vivo*

The new-indocyanine-green dye, also termed IR-820, is a lipophilic fluorescent dye in the spectral range of indocyanine-green and was used in a collagen-induced arthritis (CIA) rat model for near infrared (NIR) imaging of inflammatory conditions. Here dPGS was conjugated using an azide-terminated C11 alkyl linker and propargylamine-functionalised IR-820 (dPGS-IR-820; Figure 3; Licha et al. 2011). During *in vivo* imaging, dPGS-IR-820 was located in the joints of rats with CIA exhibiting maximum intensity at periods of 1 hour and nearly up to 3 hours post i.v. injection (Licha et al. 2011). Animals for histological evaluation were given an additional i.v. dose of dPGS conjugated with the same kind of linker to indocarbocyanine propargylamine (dPGS-ICC(1); Figure 3). Histological evaluation was conducted for liver and joints (tibiotarsal articulations) from tissues *ex vivo* 3 hours post injection (p.i.). The conjugated dPGS-ICC(1) was again found in inflamed joints, but also in the liver of CIA and healthy animals; located in macrophages (i.e., Kupffer cells), activated endothelial cells, and hepatocytes (Licha et al. 2011).

In another study the qualitative tissue uptake of ICC N-hydroxysuccinimide (NHS) ester conjugated to the amino function of dPGS amine (dPGS-ICC(2); Figure 3) was determined for the liver, spleen and kidney of healthy mice 3 hours after i.v. administration. In these healthy mice, dPGS-ICC(2) was observed in Kupffer cells and sinusoidal endothelial cells of the liver but not in hepatocytes. In the spleen, dPGS-ICC(2) was located in macrophages within the marginal zone and in endothelial cells of the blood sinusoids. The conjugate dPGS-ICC(2) was also detected in the kidney, but no further investigation was conducted to determine the specific cellular structure (Gröger et al. 2013).

In conclusion, the distribution of ICC-labelled dPGS was not completely similar, but irrespective of the type of linkage used and the state of health of the animals, dPGS was found in cells of the MPS 3 hours post dose. Furthermore, the dPGS-IR-820 conjugate exhibited a high degree of aggregation in PBS *in vitro* (Licha et al. 2011), which is known to be induced in bioconjugates by lipophilic indocyanine dyes (Biffi et al. 2013), and which has

not been seen for unlabelled dPGS with a comparable DB and DS (Weinhart et al. 2011b). This suggests altered physical properties of the NP. It is commonly accepted that the behaviour of a NP is greatly influenced by its size as well as its surface charge and hydrophobicity (Cedervall et al. 2007b). The conjugation with a dye causes substantial changes in the chemical structure and modifies these characteristics, thereby changing the interaction with proteins, leading to a different composition of the protein corona and the mode of operation of the NP (Marquis et al. 2009; Müller et al. 1997). This is particularly true for small NPs such as dPGS. The addition of an ICC molecule with a molecular weight of 767 Da or an IR-820 molecule with a molecular weight of approximately 849.47 Da increases the size of a dPGS particle, which itself only possesses a molecular weight of 10,050 Da (Gröger et al. 2013), to a considerable extent. This raises questions about the relevance of the results and requires verification.
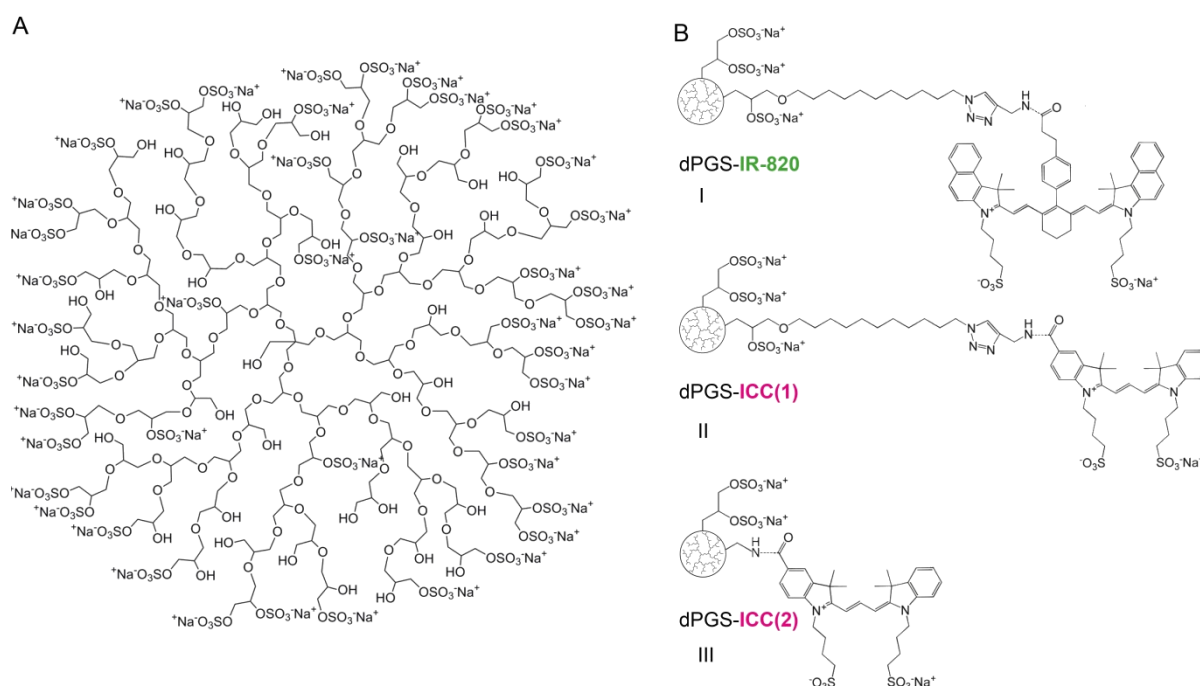
Figure 3: **A** The polymer dPGS and **B** dPGS pictogram conjugated with **I** IR-820 or **II**, **III** ICC. Modified from Gröger et al. 2013 and Licha et al. 2011. For **I** and **II** dPGS was prepared for conjugation as 11-Azidoundecanyl-polyglycerolsulfate and subsequently conjugated with **I** IR-820 propargylamine to dPGS-IR-820 or **II** ICC propargylamine to dPGS-ICC(1); for **III** dPGS was functionalised as dPGS amine and subsequently conjugated with ICC NHS to dPGS-ICC(2)

The fact that dPGS conjugates were detected in MPS organs of animals with arthritis and healthy animals alike raised concerns about non-specific targeting of this NP. Since

accumulation of dPGS in these tissues might lead to adverse effects and could diminish the long-term applicability of the NP, a histopathological analysis of the respective site of NP localisation was required. Unfortunately, fluorescent labelling does not allow cellular morphology to be evaluated (Ostrowski et al. 2015), and no information concerning this issue could be obtained from the studies using fluorescently labelled dPGS. These demands required LMA as performed in this study.

5.1.1.2 Light Microscopic Autoradiography for the Detection of ^{35}S-Labelled dPGS amine

LMA is an autoradiographic technique that records radiation in solid samples, such as histological slides, and provides a spatial localisation of a radiolabelled compound.

5.1.1.2.1 The Choice of Radioisotope

In this study LMA was applied to histological slides from mice injected with partially aminated, ^{35}S-labelled dPGS (dPG^{35}S amine). As a low beta emitter, ^{35}S has an energy of 0.167 MeV and a half-life of 87.4 days, rendering it convenient for laboratory handling, highly sensitive quantification and high resolution autoradiographic imaging (Baker 1989). Furthermore, the use of ^{35}S as a labelling agent offers a time frame that facilitates experimental settings for several weeks, allowing for long exposure times and a high sensitivity (L'Annunziata and Kessler 2012; Solon 2015). Other radioisotopes such as ^{3}H or ^{14}C may certainly have been applied as well. Tritium, however, has the disadvantage of being prone to radiolysis during storage (Baker 1989) and is known to undergo hydrogen-exchange with water *in vivo*, possibly leading to a loss of ^{3}H (Solon 2015). The isotope ^{14}C, by contrast, with its long half-life (Baker 1989) might complicate disposal. As described above, the labelling of dPGS with the radioisotope ^{35}S was performed in an efficient synthetic route prior to the start of this study (Gröger et al. 2013). Instead of the SO$_3$ pyridine complex, a ^{35}SO$_3$ pyridine complex was used as sulfation agent during synthesis. The NP used in our study was therefore chemically identical to its inactive compound (Gröger et al. 2013). *In vitro,* dPGS remained stable over 4 weeks in PBS buffer at a temperature of 37°C and pH 7.4, pH 7.4 with addition of carboxylesterase 1, or pH 5.0 (Reimann et al. 2015). Cleavage of the sulfur esters of dPGS *in vivo* therefore seems unlikely and a stable conjunction of radioisotope and specimen can be assumed.

5.1.1.2.2 Implementation of Light Microscopic Autoradiography

We modified the LMA by reducing the section thickness of the paraffin wax sections. In doing so standard histological techniques could be employed to improve the histological quality and no special procedures had to be applied such as resin sections (Baker 1989; Holzhausen et al. 2013). Furthermore, coating of the tissue sections with the emulsion was conducted after staining to reduce artefacts from the dehydration and hydration processes. In addition, when

the sections still wet from staining, better adhesion of the galantine-based emulsion was obtained. For final coverage Kaiser´s glycerol was utilised as a hydrophilic adhesive to prevent disappearance of grains in the longer term (Baker 1989; Holzhausen et al. 2013).

Applied to tissue sections from mice treated with dPG^{35}S amine, LMA provided images with a resolution of approximately 0.2 µm when using Ilford nuclear emulsion K5D, thus making it possible to obtain a detailed biodistribution profile that included an assignment to cells. We were able to localise the particles, in order of the ascending time bar, 2 minutes p.i. in the glomerular capillary or 1 hour p.i. in the proximal tubulus epithelial cells of the kidney, 24 hours p.i. in sinusoids and hepatocytes, and up to 21 days in the Kupffer cells of the liver, the marginal zone of the spleen, etc. (Holzhausen et al. 2015). An exact assignment to subcellular structures by LMA was not possible. A solution to this issue could possibly be attained by electron microscopic autoradiography (Solon 2015).
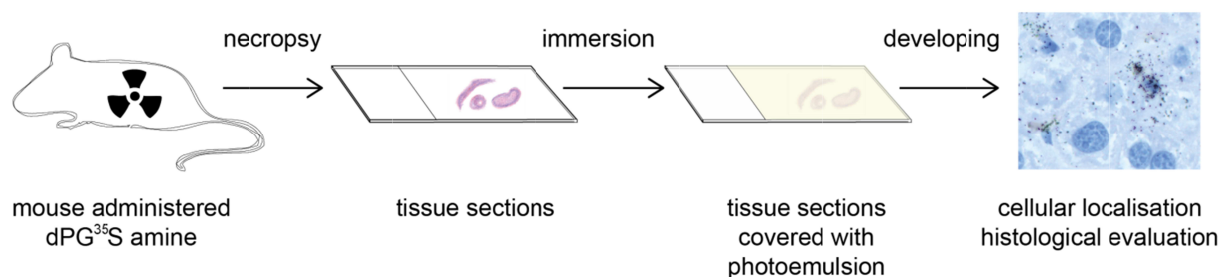
Figure 4: Principle sketch of processing light microscopic autoradiography (LMA). Source Cornelia Holzhausen

Besides being based on radioactive labelling and therefore enabling the detection of the unadulterated cellular localisation of an NP, the other, outstanding advantage of LMA is the opportunity it presents to simultaneously evaluate the surrounding cells in a routine standard histopathological examination (Holzhausen et al. 2013). In the light of dPGS accumulation in the cells of the MPS, a simultaneous histopathological analysis is particularly valuable and was conducted to assess the general toxicity of dPGS (see section 5.1.4 below). As soon as future research has solved the current drawbacks of LMA, namely the costs and the low throughput limits (Solon 2015), it will offer a promising approach to determining cellular localisation and to evaluating the histopathological changes following NP treatment in basic research.

5.1.2   Aim No. 2: Perform a kinetic study that reveals the time-dependent tissue concentrations, cellular localisation, and excretion as well as the terminal blood half-life of radioactive dPG^{35}S amine in the major organs after i.v. or s.c. application to mice

5.1.2.1   *In vivo* Biokinetic Behaviour of dPGS amine for i.v. Mode of Application

The major target organs of dPG^{35}S amine in this study were the liver and spleen, which can easily be explained by their affiliation to the MPS. In these organs the dPG^{35}S amine concentration, as measured by LSC, increased over time and according to LMA was present even after 21 days in the Kupffer cells of the liver and the red pulp of the spleen (Holzhausen et al. 2015). Furthermore, persisting concentrations of dPG^{35}S amine were found in analysis of the femur together with the bone marrow it contained up to day 21 p.i. (Holzhausen et al. 2015). In an *in vitro* study using dPGS-rhodamine B, it was presumed that dPGS binds weakly to hydroxyapatite in native bone sections and only in demineralised bone matrix to collagen (Gröger et al. 2014). Hence it is more likely that the persisting dPG^{35}S amine concentration in this study was related to the existence of MPS cells within this tissue. This is consistent with the detection of ^{3}H dPGS in the bone marrow (Pant et al. 2015). One important factor that determines the ability of a compound to accumulate in the MPS is its potential to be excreted.

In the urine, dPG^{35}S amine was found in higher concentrations only at the first time points p.i. (2 minutes, 15 minutes) decreasing by 24 hours p.i. to almost zero. According to the literature, particles with an HD below 6 nm are in fact rapidly cleared by the kidney; this leads to a short blood half-life and low uptake in MPS organs (Choi and Frangioni 2010; Longmire et al. 2008). For dPGS amine, with an HD of 5.7 nm ± 1.5 nm, the close proximity to the glomerular filtration threshold together with the negatively charged sulfate groups that repel the dPG^{35}S amine from the negatively charged glomerular basal membrane (Longmire et al. 2008) and their high degree of branching may cause restricted filtration (Choi and Frangioni 2010; Lee et al. 2005). Apart from that, dPGS amine with its additional functional amino groups was found to experience partial aggregation in PBS under physiological pH of 7.4 as measured by DLS (Gröger et al. 2014), possibly hindering filtration. The assumption of aggregation was supported by a zeta potential of dPGS amine (-31 ± 6 mV) which unexpectedly was more negative than for the un-functionalised dPGS with comparable size, DB and DS and lies in the range between small (6 nm, 13 kDa, -13.6 ± 3.1 mV) and larger (12 nm, 250 kDa, -34.3 ± 4.7 mV) dPGS macromolecules. Un-functionalised dPGS with equivalent DB and DS did not exhibit aggregation in PBS (Gröger et al. 2013; Weinhart et al. 2011b). Follow-up studies with i.v. injection of ^{3}H-radiolabelled dPGS without amino functionalities displayed an organ distribution profile similar to dPG^{35}S amine after i.v. injection (Pant et al. 2015). Hence the restriction of renal filtration of dPG^{35}S amine does not

seem to be attributable to the few amino functionalities. Interestingly, when LMA was used on kidney sections, apart from being found in the glomerular capillaries and the tubular lumen 2 minutes and 15 minutes p.i. respectively, dPG^{35}S amine was also found in the proximal tubular epithelial cells 1 hour following application, which suggests a reabsorption (Holzhausen et al. 2015). Reabsorption partially negates glomerular filtration and may also explain the biphasic decrease of the dPG^{35}S amine concentration in the kidney as measured by LSC. In contrast to other organs, the concentration in the kidney is not only related to the amount of dPG^{35}S amine in the parenchyma, the concentration in the blood and the perfusion of the organ, but also to the enrichment of dPG^{35}S amine associated with urine production. Anion transporters play a major role in both secretion and reabsorption of anionic compounds by renal epithelial cells (Van Aubel et al. 2000). It therefore seems likely that such an active mechanism is also relevant for the transport of the polyanionic dPG^{35}S amine.

In addition to renal filtration, excretion of dPG^{35}S amine via hepatobiliary clearance is likely, as indicated by a low but persisting amount of the polymer that was detected in the faeces by LSC and which corresponds to the generally slow hepatic processing (Longmire et al. 2008). Hepatobiliary clearance is further supported by the high concentrations in the liver measured 24 hours p.i., decreasing towards the time point of 21 days. Accordingly, in the corresponding tissue sections evaluated by LMA, dPG^{35}S amine was associated with sinusoids, hepatocytes and Kupffer cells at 24 hours p.i whereas at 21 days p.i. it was solely found in Kupffer cells. Two routes of elimination via the liver seem possible. Either directly, when dPGS amine is taken up by hepatocytes or via the metabolism of Kupffer cells with subsequent elimination. The accessibility of hepatocytes is determined by the size of fenestrations in the sinusoid epithelium which is 140 nm in mice (Moghimi et al. 2001; Wisse et al. 2008). Besides this mere geometrical limitation of size, the route of the NP is determined by its protein corona (Walkey and Chan 2012).

Following incubation of dPGS with human plasma for 5 minutes at 37°C, the proteins albumin, fibrinogen γ and β chains, and apolipoprotein A-1 (apo A-1) were the major proteins found to be adsorbed to dPGS *in vitro* (Staufenbiel et al. 2014). The latter, protein apo A-1, is the major protein component of high-density lipoprotein (HDL) in plasma. It promotes the transport of lipids (including cholesterol) from tissues to the liver for excretion. In the liver it mediates the uptake of cholesterol through the scavenger receptor B1 (SRB1; (Canton et al. 2013). The cholesterol delivered to the liver is excreted into the bile and hence intestine, which is a route the NP dPG^{35}S amine might have taken as well. Albumin is a dysopsonin and increases the ability of a particle to hide from the MPS cells, and therefore, the potential of direct excretion. Fibrinogen, in contrast, has opsonin activity (Staufenbiel et al. 2014). As described above, opsonins enhance the recognition of particles by cells of the MPS, resulting in a fast uptake within minutes. For dPG^{35}S amine, uptake in MPS cells was only apparent by

LMA after 24 hours. Staufenbiel and co-workers believed the heterogeneity of the NP surface properties, among other factors, to be responsible for the different types of proteins absorbed and the resulting kinetics of dPGS after i.v. injection. The proteins enable one fraction of dPG^{35}S amine to avoid recognition and immediate clearance from the blood-stream, and thus to experience excretion via the liver, whereas another fraction is susceptible to uptake by macrophages. For dPGS amine taken up by macrophages (e.g. Kupffer cells) this means, if it is non-biodegradable by lysosomes and subsequently eliminated via the liver, accumulation and long-term retention will occur. Analysis with LMA in this study supports the presumed retention, because, as mentioned above, dPG^{35}S amine was found in MPS cells at least up to 21 days p.i. in a noticeable amount.

In other organs such as the lung, intestine, testes, and brain dPG^{35}S amine displayed an overall distribution pattern that reproduces the clearance from the blood, with the highest concentrations being found 2 minutes p.i. and a continuous decrease thereafter (Holzhausen et al. 2015). However, in the testes only low amounts of dPG^{35}S amine were measured, and in the brain the polymer was only marginally detectable. It has to be noted that, as mentioned above, mice were not exsanguinated after euthanasia. Consequently the blood-tissue barrier, especially in the brain, seems tight for this polymer. Nevertheless, apart from in the liver, the SRB1 is also located in the blood-brain barrier (BBB). The absorption of apo A-1 to dPGS was therefore assumed to enhance the brain uptake in an *in vitro study* (Staufenbiel et al. 2014). Yet, this study did not find dPG^{35}S amine within the tissue of the brain at any time point or with any of the techniques applied (Holzhausen et al. 2015). This finding is supported by ^{3}H-labelled dPGS without amino function (Pant et al. 2015). As a consequence, this *in vitro* finding of Staufenbiel and co-workers needs further investigation *in vivo*. The most likely explanation is that the protein apo A-1 alone is not capable of promoting an uptake across the BBB.

The apparent terminal blood half-life for dPG^{35}S amine was calculated as approximately 12 days. This is considerably longer than half-lives reported for other polymers such as hyperbranched polyglycerol (Kainthan and Brooks 2007; Yamaoka et al. 1994). The definition of half-life is often understood in different ways, which may lead to misinterpretations. In this study, the "terminal blood half-life" was specified. For a multi-compartment (open model) pharmacokinetic, at least two phases can be distinguished after i.v. injection. The first, initial phase, in which the blood concentration decreases steeply, is primarily determined by distribution into tissues and simultaneous elimination. In the second phase, which is less steep, the theoretical equilibrium of the compound in the tissues with that in blood is achieved gradually. Finally, the decline of the concentration in the blood is associated solely with the elimination from the body. The terminal half-life refers to the time when half of the dose has been eliminated from the vasculature during the elimination phase

(Toutain and Bousquet-Mélou 2004). The half-clearance, on the contrary, specifies the time when half of the initially administered dose has been cleared from the vasculature; for dPG^{35}S amine this time was about 15 minutes. In a subsequent study by Pant and co-workers this time for ^{3}H dPGS with a similar DB and DS was between 15 and 30 minutes. The terminal half-life has the advantage of providing information about the length of time a compound is retained within the organism (e.g. in the MPS), because it considers redistribution processes. Consequently, a long terminal half-life predicts retention of a compound (Toutain and Bousquet-Mélou 2004). The half-clearance from the blood, on the other hand, can actually be very short due to the uptake by the cells of the MPS (Staufenbiel et al. 2014; Walkey and Chan 2012).

Pant and co-workers observed a distribution and concentrations that were generally similar using ^{3}H-labelled dPGS with a comparable DS and DB (Pant et al. 2015), confirming the advantage of radioactive labelling in obtaining a reliable biodistribution. Yet, a discrepancy exists for the data of the organ distribution prior to the time point of 24 hours. This deviation is quite likely attributed to the exsanguination performed in the quoted study in contrast to the study presented here. The absence of exsanguination in this study is explained by the aim to perform a systematic state-of-the-art toxicopathological analysis. It was therefore of interest to also examine blood cells in the blood vessels to investigate aspects such as leucocyte trafficking, inflammation-associated blood congestion or hyperaemia.

5.1.2.2 *In vivo* Biokinetic Behaviour of dPG^{35}S amine for s.c. Mode of Application

In addition to the i.v. injection of dPG^{35}S amine, s.c. application to mice was also performed. This mode of administration was carried out because it might be of interest for dPGS as a therapeutic for self-administration by outpatients with chronic conditions.. In addition, in the field of rheumatoid arthritis, a prolonged release or the formation of a local drug depot can be important. Furthermore, the use of subcutaneously administrated dPGS as drug carrier could be of use in anti-cancer therapy, especially for targeting the lymph nodes. With a view to anti-cancer therapy, the dPGS polymer has recently been loaded with the drug paclitaxel (Sousa-Herves et al. 2015).

In general, dPG^{35}S amine showed a delay in its biodistribution after s.c. application. This can be explained by the slow availability from the s.c. injection site. The maximum concentration in the blood was only reached 1 hour following application. In the case of s.c. application, no clearance via the urine was observed. After 24 hours, however, the concentrations in the organs were very close to those found after i.v. injection (Holzhausen et al. 2015). The delayed availability following s.c. application does, therefore, not seem to influence the velocity and magnitude of MPS targeting. The delayed biodistribution may be of interest in

cases where a slow release is desired. However, the s.c. mode of application is not suitable for diagnostic purposes.

5.1.3 Aim No. 3: Provide first evidence towards the potential acute and subacute general toxicity of dPGS on the basis of macropathology and histopathology

No adverse clinical signs were observed during monitoring conducted throughout the course of the experiment. Examination of the carcass did not show any gross lesions. Furthermore, histopathological evaluation did not reveal any toxicity in the tissues examined, neither within the vicinity of the dPG^{35}S amine localisation as determined with LMA, nor due to radiation. Thus, from a pathomorphological point of view, application of dPGS amine appears tolerable under the circumstances tested.

5.2 Applicability of dPGS for Biomedical Purposes (Therapy, Diagnostics)

In addition to the use as a selectin blocker or inflammation-modulating agent (Budde et al. 2015; Maysinger et al. 2015; Schneider et al. 2015), dPGS could be used as carrier for drugs. A first attempt in this field was recently undertaken with the linkage of dPGS to the anti-cancer drug paclitaxel (Sousa-Herves et al. 2015). Although the ester linkage used in this approach was reported to lead to a very fast release of the drug, which was undesirable in the anti-cancer setting (Sousa-Herves et al. 2015), ester linkage could be used in other processes requiring fast drug release or cleavage (see below).

As mentioned above, dPGS is also under investigation as a diagnostic tool. The applicability of fluorescent dPGS-IR-820 was investigated for two different medical imaging modalities, namely NIR fluorescent imaging (Licha et al. 2011) as well as optoacoustic tomography for the imaging of rheumatoid arthritis (Beziere et al. 2014). The conjugated particles still had selectin-blocking properties, leading to a bifunctional particle for concurrent therapy and imaging (Licha et al. 2011). In a recent study a similarly combined functionality was reported for the use of dPGS functionalised iron oxide NPs in magnetic resonance imaging (MRI) analysis of inflamed tissue and yielded promising results (Nordmeyer et al. 2014).

However, even 21 days after administration dPG^{35}S amine particles were still detected in the MPS. Although toxic effects have not unambiguously been proven, the uptake of dPGS polymers by the MPS is undesirable. Apart from the risk of long-term toxicity, accumulation in the MPS limits the use of dPGS as a contrast agent. In the case of NPs as diagnostic tools, it has to be ensured that they do not interfere with other imaging techniques or with repeated examinations (Choi and Frangioni 2010; Longmire et al. 2008). Moreover, NPs that are

primarily cleared from the blood by MPS cells are specifically limited in their utility to diagnose inflammatory and degenerative disorders where macrophages are involved in higher numbers (Choi and Frangioni 2010). For one thing synovial macrophages, for example, are numerous in the inflamed joint and play an important role in rheumatoid arthritis (Kinne et al. 2000). Uptake into macrophages may have led to the enhanced signals in the joints of the CIA animal model after i.v. administration of fluorescently labelled dPGS (Licha et al. 2011) and not the selectin binding.

One way to avoid accumulation is the use of differential protein adsorption patterns of NPs. This concept includes modification of the NP surface in a way that leads to selective adsorption of the plasma proteins required for targeting a certain tissue or cell (Staufenbiel 2014). Smaller, uncharged ^{3}H-labelled dPG (i.e. "pure" polyglycerol), for example, were excreted via the urine to a high degree while no uptake in MPS organs was seen (Pant et al. 2015). This confirms the presumed responsibility of size and especially of the charged terminal groups for this branched polymer regarding MPS uptake. Unfortunately uncharged polyglycerol without sulfation does not possess anti-inflammatory properties (Türk et al. 2004), thus foiling surface modifications in this direction. As described above, the surface of dPGS was found to be covered, among other substances, by two proteins: albumin which is thought to hinder uptake by macrophages, and fibrinogen which increases opsonisation and thus uptake by the MPS (Staufenbiel et al. 2014). These two proteins might compete for the surface of the NP leading to different distributions in the body. Sophisticated modifications of the surface properties of dPGS to decrease adsorption of fibrinogen γ and β chains might prove efficient in decreasing uptake of the particle by the MPS. However, information on how precisely surface properties attract certain proteins still requires substantial research. Any changes in the surface characteristics may lead to a modified biodistribution and further studies would be required to determine the distribution of such a modified dPGS.

Another way may be a degradable or shell cleavable dPGS where the sulfated, charged outer shell is released due to enzymatic cleavage or an acidic pH within a cellular compartment (e.g. in lysosomes), resulting in neutral dPG and multiple sulfated moieties. In a recent *in vitro* study, different degradation patterns based on ester, amide or carbamate were introduced. The potentially biodegradable dPGS still exhibited anti-inflammatory properties of different degrees in the selectin binding assay, the complement activation test and the aPTT clotting assay. The cleavage, however, was of varying efficacy and though some linkers showed promising results, no complete decomposition could be obtained (Reimann et al. 2015). The monitoring of such an approach will need a study design that allows for a separate tracing of the resulting components. One way could be to use radioisotopes emitting different nuclear decay energies, such as ^{3}H and ^{35}S, and then employ LSC, for example, to determine the fate of the cleavage products *in vivo*.

This study was performed using healthy mice, which did not exhibit any type of inflammation. As mentioned, dPGS blocks selectin-mediated adhesion of inflammatory cells by occupying the binding sites of P-selectin and L-selectin (Dernedde et al. 2010). The distribution in healthy animals is not necessarily similar to that in animals with any type of inflammation. In the latter, dPGS interacts with selectins. Furthermore, employing a direct binding assay, dPGS was found to bind the complement factor C3 (Dernedde et al. 2010), which is a common opsonin upon cleavage. The absence or presence of inflammation might influence the quantity and quality of adsorption and hence the degree of opsonisation. In the context of inflammation therefore, dPGS might be distributed in the body in a completely different manner, either including or excluding storage in the MPS. A first hint that uptake in the MPS is not prevented by inflammation was given by a study already quoted above for *in vivo* NIR fluorescent imaging where, in an animal model of arthritis, IR-820-labelled dPGS was not only found in the joints but also in liver macrophages (i.e. Kupffer cells, Licha et al. 2011).

The study presented here provides a good basis for further dose-finding as well as repeated-dose studies and other studies evaluating the safety of dPGS NPs for biomedical applications. Apart from the issue of MPS storage of dPGS amine, this study did not discover any impediments to hamper the further development of dPGS with a view towards therapeutic or diagnostic usage.

6 CONCLUSION AND OUTLOOK

The incorporation of the radioisotope ^{35}S has proven valuable in assessing the kinetics of dPGS amine. Comparable results for the distribution of the NP were obtained in a subsequent study using ^{3}H-labelled dPGS. Hence, radiolabelling may also contribute to understanding the kinetics of other NPs and promote their development. Furthermore, the LMA technique offered high resolution cellular localisation data for dPG^{35}S amine, and thus allowed for a more detailed view of the biodistribution compared to the assessment of quantitative tissue concentrations alone. In addition, this technique also enabled a concurrent histopathological evaluation of these cells. LMA may also be used for other soft NPs to deliver important information about their fate in cells and the morphology of such cells in basic research.

The major target organs of dPG^{35}S amine were liver and spleen. In these organs the polymer accumulated 24 hours after administration for both the i.v. and s.c. mode of application but later, at the time point of 21 days, had partially decreased. This decrease in particle concentration, together with the discovery of low dPG^{35}S amine concentrations in the faeces, suggests slow hepatobiliary excretion. Elimination via the liver can take place either directly by hepatocytes or via uptake into macrophages (i.e., Kupffer cells) and subsequent metabolisation. If not readily degraded in the lysosomes of the macrophages, dPGS amine becomes sequestered. LMA supports the latter, because dPG^{35}S amine particles were still observed very clearly in association with macrophages up to the last point in time. Hence, as was the case with dye-labelled dPGS, radioactive dPGS was also found in cells of the MPS. It may therefore be assumed that dPGS can be recognised by macrophages and is thus retained in the body. Furthermore, the assumption of such retention in the body was confirmed by the long apparent terminal blood half-life of dPG^{35}S amine, which exceeded 12 days. In addition to hepatobiliary clearance, dPG^{35}S amine was excreted via the kidneys but only for the i.v. mode of application and at early time points.

Retention in the body, e.g. accumulation in the MPS, is undesirable for any drug, because it may cause long-term toxicity and can interfere with diagnostic imaging applications. Whether or not and how fast a particle is taken up by macrophages might be controlled by the composition of the protein corona adsorbed onto its surface. Knowledge about the relation of the physicochemical parameters of the NP and the resulting protein absorption is still limited and hinders a target-oriented surface design. Future attempts to develop dPGS for biomedical applications should nevertheless consider the surface design, also in terms of any resulting protein pattern, to avoid uptake by MPS cells.

No clinical signs or pathomorphological changes were found that would impede further investigations of dPGS for therapeutic and diagnostic usage. However, the approval of dPGS NP as a candidate drug will need further pharmacokinetic and toxicological analysis according to the ICH M3(R2) guidance document. With regard to the retention of dPGS amine in the organism and the desired application as an anti-rheumatic that will be taken repeatedly and in the long run, dPGS will need to be assessed for long-term application with chronic and repeated-dose toxicity testing that is consistent with the ICH guidelines. In view of the special properties of NPs in general and dPGS in particular, the investigation of additional parameters, which are not typically anchored in the guidelines, such as possible aggregation effects *in vivo*, might also be useful. In addition, attention should also be paid to the potential difference in the distribution between healthy animals and those animals with any type of inflammation because here dPGS interacts with selectins or other components of inflammation which may modify the behaviour. Future investigations will also have to address this important issue.

7 SUMMARY

Anti-Inflammatory Dendritic Polyglycerol Sulfate Nanoparticle: Biodistribution, Elimination, Cellular Localisation and Toxicopathology in Mice

Cornelia Holzhausen

The prefix "nano" refers to any material smaller than 100 nm in one dimension. Materials with all dimensions in the nanoscale are defined as nanoparticles (NPs). At this dimension quantum effects unfold and nanomaterials (NMs) show an enhanced reactivity due to their high area-to-mass ratio. Many opportunities for innovations in industry and biomedicine originate from the unique features of NMs. In biomedicine their applications include medical devices, in *vitro* and *in vivo* diagnostics, drug delivery and therapeutics. A promising NP for different biomedical applications, in which the most prominent are the diagnosis and therapy of inflammation, is the dendritic polyglycerol sulfate (dPGS). However, the unique features of NMs can also lead to unforeseeable behaviour and may bear safety risks.

Problems in risk management of NMs are in particular the missing consensus and availability of methods adequate for safety testing. Furthermore, to date specific regulatory guidance has been released for only a few NMs. The vast majority of NMs are regulated in preclinical approval like "regular" small drugs in accordance with the guidance document M3(R2) of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. With reference to M3(R2), preclinical drug approval includes pharmacology, toxicokinetics and pharmacokinetics as well as toxicity studies. Yet further parameters, related to the unique features of NMs, such as particle size or aggregation effects, may be needed for risk assessment. Moreover, standard tests can interfere with NMs and require verification.

The study presented here surveys the kinetics as well as the acute and subacute toxicopathology of dPGS and thus contributes to providing a basis for further research with a view towards drug approval. In previous *in vivo* efficacy studies fluorescently labelled dPGS showed no obvious acute adverse effects. The assessment of dPGS for biomedical applicability has so far been promising, but no systematically surveyed data regarding the distribution, elimination and histopathology of healthy animals, as common determinants in preclinical safety assessment, were available. Furthermore, fluorescent labelling is thought to alter NP properties leading to a different biodistribution, and hence requires verification. In addition, fluorescent labelling does not allow for histopathological evaluation. Techniques feasible to trace "soft" NPs such as dPGS *in vivo* are, however, limited. Due to their small size, all NPs escape the wavelength of light and require labelling for light microscopy, while the low atomic number of "soft" NPs also rules out electron microscopy. There are innovative methods, such as Raman microspectroscopy, which do not require labelling and allow NPs

to be localised in cells and enable cellular alterations to be evaluated, but have the disadvantage of being technically demanding and not widely available. An alternative to labelling with fluorophores is labelling with radioisotopes. Provided the isotope is well integrated, the chemical structure of the NP is preserved. The radiation can be recorded by whole body/organ autoradiography (WBA/WOA), but can also be used for quantitative kinetic studies employing quantitative whole body/organ autoradioluminography (QWBA/QWOA) or liquid scintillation counting (LSC). Yet, these techniques only provide data on tissue level. To detect radioactively labelled NPs in cells, the light microscopic autoradiography (LMA) was established in this study. LMA also allows for concurrent histopathological analysis.

The radioisotope ^{35}S was incorporated into the dPGS NP specifically for this study. This isotope was particularly suitable because sulfur is a constituent of dPGS. It should be noted that amino functions were integrated for easy conjugation with a drug or dye. The resulting radioactive dPG^{35}S amine was administered i.v. or s.c. to healthy mice. The mice were sacrificed but not exsanguinated at different time points following application, and samples were collected for LSC or autoradiography (WOA, QWOA, and LMA). The dPG^{35}S amine concentration in liver and spleen increased up to 5 and 21 days following i.v. application respectively. Evaluation of tissue sections with LMA localised dPG^{35}S amine in the Kupffer cells of the liver and in the red pulp of the spleen 24 hours, 5 and 21 days post dose. In other organs such as the kidney, lung, intestine, testes, and brain the overall dPG^{35}S amine concentration decreased over time. Only low concentrations were measured in the testes and marginal concentrations in the brain, suggesting a tight blood-tissue barrier for this NP. Furthermore, dPG^{35}S amine was found in the faeces and at early time points in the urine. Taken together these data do indeed suggest a partial elimination via the liver and kidney, but apart from that dPG^{35}S amine is retained in the Mononuclear Phagocyte System (MPS). Particles that are not readily degraded in macrophages become sequestered, as confirmed by LMA for dPG^{35}S amine. The long apparent terminal blood half-life of dPG^{35}S amine, exceeding 12 days, also indicates retention. Apart from the delayed onset, the distribution of this NP to the organs after s.c. application was very similar to its i.v. application.

In this study neither the clinical evaluation during the experiment nor the gross and histopathological analysis of the examined tissue showed any adverse effects. Thus, from a pathomorphological point of view, there was no evidence that would impede future investigations of dPGS for biomedical usage. Accumulation of dPGS in MPS cells, which is known for other charged NPs as well and depends on the protein corona of the NP, however, always bears the risk of toxicity and hinders an application as a therapeutic or diagnostic agent. In addition, retained NPs may interfere with diagnostic imaging. The retention of dPGS in MPS cells will have to be addressed using long-term and repeated-dose toxicity testing as well as in any further attempts to develop dPGS for biomedical applications.

8 ZUSAMMENFASSUNG

Entzündungshemmender dendritischer Polyglycerol Sulfat Nanopartikel: Bioverteilung, Ausscheidung, zelluläre Lokalisierung und Toxikopathologie bei Mäusen
Cornelia Holzhausen

Die Vorsilbe „Nano" bezeichnet alle Materialen kleiner als 100 nm in einer Dimension. Materialien mit allen Abmessungen im nanoskalaren Bereich werden als Nanopartikel (NPs) bezeichnet. In dieser Größenordnung treten Quanteneffekte zum Vorschein. Zudem zeigen Nanomaterialien (NMs) als Folge ihres hohen Fläche-zu-Masse-Verhältnisses eine erhöhte Reaktivität. Aus den besonderen Eigenschaften von NMs ergeben sich viele Chancen für Innovationen in Industrie und Biomedizin. In der Biomedizin umfasst ihr Einsatzbereich Medizinprodukte*, in vitro* und *in vivo* Diagnostik, Arzneimittelträger und Therapeutika. Ein vielversprechender NP für verschiedenste biomedizinische Anwendungen, von denen die größte Bedeutung in der Diagnose und Therapie von Entzündungen liegt, ist das dendritische Polyglycerol Sulfat (dPGS). Die besonderen Eigenschaften von NMs können jedoch auch zu unvorhersehbarem Verhalten führen und ein Sicherheitsrisiko beinhalten.

Probleme beim Risikomanagement von NMs sind insbesondere der fehlende Konsens und die fehlende Verfügbarkeit von geeigneten Prüfmethoden. Außerdem sind bis heute nur für wenige NMs spezifische regulatorische Leitlinien erlassen worden. Für die präklinische Zulassung wird der überwiegende Anteil der NMs genau wie „reguläre" niedermolekulare Arzneimittel reguliert. Dies erfolgt gemäß der Leitlinie M3(R2) des Internationalen Rates für Harmonisierung technischer Anforderungen an Pharmazeutika für den menschlichen Gebrauch. Entsprechend M3(R2) beinhaltet die präklinische Arzneimittelzulassung: Pharmakologie, Toxi- und Pharmakokinetik sowie Untersuchungen zur Toxizität. Für eine Risikoeinschätzung könnten jedoch weitere Parameter benötigt werden, welche die besonderen Eigenschaften wie beispielsweise die Partikelgröße oder Aggregationseffekte von NMs berücksichtigen. Darüber hinaus können Standardtests mit NMs interferieren und müssen verifiziert werden.

Die vorliegende Studie untersucht die Kinetik sowie die akute und subakute Toxikopathologie von dPGS und trägt somit zu einer Grundlage für weitere Forschung in Richtung Arzneimittelzulassung bei. In vorangegangenen Wirksamkeitsstudien gegen Entzündungen zeigten fluoreszenzmarkierte dPGS *in vivo* keine offensichtlichen akuten Nebenwirkungen. Soweit war die Beurteilung der biomedizinischen Anwendbarkeit von dPGS sehr vielversprechend, jedoch lagen keine systematisch erhobenen Daten gesunder Tiere vor, welche die Verteilung, Ausscheidung und Histopathologie als übliche Bestimmungsgrößen einer präklinischen Sicherheitsbewertung berücksichtigen. Außerdem wird angenommen,

dass eine Fluoreszenzmarkierung die Eigenschaften eines NP verändert, so zu einer abweichenden Bioverteilung führt und folglich eine Verifikation erfordert. Darüber hinaus erlaubt die Fluoreszenzmarkierung keine histopathologische Beurteilung. Geeignete Techniken, um „weiche" NPs wie dPGS *in vivo* nachzuweisen, sind allerdings nur begrenzt vorhanden. Aufgrund ihrer geringen Größe entziehen sich alle NPs der Wellenlänge des Lichts und benötigen eine Markierung, um unter dem Lichtmikroskop sichtbar zu sein. Die niedrige Atomzahl „weicher" NPs schließt jedoch auch den Einsatz der Elektronenmikroskopie aus. Es gibt innovative Methoden, wie beispielsweise die Raman-Spektroskopie, die keine Markierung benötigen, mit denen man NPs in Zellen lokalisieren sowie zelluläre Veränderungen beurteilen kann. Diese haben jedoch den Nachteil, technisch anspruchsvoll und nicht allgemein verfügbar zu sein. Eine Alternative zur Markierung mit Fluorophoren ist die Markierung mit Radioisotopen. Sofern das Isotop gut integriert ist, bleibt die chemische Struktur des NP dabei erhalten. Die Strahlung kann mit der Ganzkörper-/Ganzorganautoradiographie (WBA/WOA) aufgezeichnet werden oder dazu verwendet werden, um quantitative kinetische Studien mit der quantitativen Ganzkörper-/Ganzorganautoradiographie (QWBA/QWOA) sowie mit der Flüssigszintillationszählung (LSC) durchzuführen. Diese Techniken stellen jedoch nur Daten auf Gewebeebene bereit. Um radioaktiv markierte NPs in Zellen nachzuweisen, wurde in diesere Studie die lichtmikroskopische Autoradiographie (LMA) etabliert. Die LMA lässt auch eine simultane histopathologische Untersuchung zu.

Das Radioisotop ^{35}S wurde eigens für diese Studie in den dPGS NP integriert. Weil Schwefel ein Bestandteil von dPGS ist, war dieses Isotop besonders geeignet. Zu beachten ist, dass funktionelle Aminogruppen für eine einfache Konjugation mit Farbstoffen oder Arzneimitteln eingefügt wurden. Das daraus resultierende radioaktive dPG^{35}S amine wurde gesunden Mäusen i.v. oder s.c. verabreicht. Die Mäuse wurden zu unterschiedlichen Zeitpunkten nach der Applikation getötet, aber nicht entblutet und Proben für die LSC und die Autoradiographie (WOA, QWOA und LMA) wurden entnommen. Die dPG^{35}S amine Konzentration in Leber und Milz stieg bis zum Tag 5 bzw. Tag 21 nach der i.v. Applikation an. Bei der Untersuchung von Gewebeschnitten mit der LMA wurde dPG^{35}S amine in den Kupffer Zellen der Leber sowie in der roten Milzpulpa 24 Stunden, 5 und 21 Tage nach seiner Verabreichung lokalisiert. In anderen Organen wie Niere, Lunge, Darm, Hoden und Gehirn nahm die dPG^{35}S amine Konzentration gesamt gesehen über die Zeit ab. Dabei wurden nur geringe Konzentrationen in den Hoden und nur marginale Konzentrationen im Gehirn gemessen, was auf eine dichte Blut-Gewebe-Schranke für diesen NP hindeutet. Des Weiteren wurde dPG^{35}S amine im Kot und zu den frühen Zeitpunkten auch im Urin nachgewiesen. Diese Daten legen zusammengenommen zwar eine partielle Ausscheidung über Leber und Niere nahe, aber davon abgesehen wird dPG^{35}S amine im Mononukleären Phagozytensystem (MPS) zurückbehalten. Partikel, die in Makrophagen nicht rasch degradiert werden, werden

sequestriert, wie es für dPG^{35}S amine durch die LMA bestätigt wurde. Auch die lange, vermutlich terminale Halbwertszeit von dPG^{35}S amine von über 12 Tagen weist auf eine Retention hin. Abgesehen von einem verzögerten Einsetzen der Verteilung dieses NP in die Organe nach s.c. Applikation, war diese der Verteilung nach i.v. Applikation sehr ähnlich.

In der vorliegenden Studie zeigten sich weder in klinischen Untersuchungen während des Experiments noch durch makro- oder histopathologische Beurteilung des untersuchten Gewebes Nebenwirkungen. Vom pathomorphologischen Standpunkt aus gibt es daher keine Hinweise, die gegen eine weitere Erforschung von dPGS für biomedizinische Zwecke sprechen. Die Anreicherung von dPGS in MPS Zellen, welche auch für andere geladene NPs bekannt ist und von der Proteinkorona des NP abhängt, beinhaltet hingegen immer das Risiko einer Toxizität, wodurch eine Anwendung als Therapeutikum oder Diagnostikum schwierig wird. Außerdem können zurückgehaltene NPs mit diagnostischer Bildgebung interferieren. Die Retention von dPGS in MPS Zellen wird daher in Toxizitätsuntersuchungen durch Langzeit- und Mehrfachapplikationen sowie in allen zukünftigen Bestrebungen, dPGS für die biomedizinische Anwendung zu entwickeln, berücksichtigt werden müssen.

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10   LIST OF PUBLICATIONS

Research publications in scientific journals:

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12 SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 07.10.2016

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