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Dendritic Core-Multishell Nanocarriers for the Delivery of Active Compounds

DISSERTATION

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Emanuel Fleige

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1. Gutachter: Prof. Dr. Rainer Haag

2. Gutachter: Prof. Dr. Christoph Schalley

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…to those who I care for

and those who care for me

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Table of Contents

Abbreviations

CAC	critical aggregation concentration
CAL B	candida antarctica lipase B
CC	*cis*-1,2-cyclohexanedicarboxylic acid
CMC	critical micelle concentration
CMS	core-multishell
cRGD	cyclic tripeptide (arginine, glycine, aspartic acid)
DDS	drug delivery system
DNA	deoxyribonucleic acid
DOX	doxorubicin
dPG	dendritic polyglycerol
DTT	1,4-dithiothreitol
EPR	enhanced permeation and retention
FDA	US Food and Drug Administration
GSH	glutathione
HIV	human immunodeficiency virus
LA	lipoic acid
LCST	lower critical solution temperature
lPG	linear polyglycerol
mPEG	mono methyl ether poly(ethylene gycol)
MPS	mononuclear phagocyte system
PAA	poly(acrylic acid)
PAAm	poly(acrylamide)
PAMAM	poly(amido amine)
PAsp	poly(aspartate)
PCL	poly(ε-caprolactone)
PDEAEMA	poly(diethylaminoethyl methacrylate)

PDEAM	poly(*N*,*N*-diethylacrylamide)
PDI	polydispersity index
PDMAEMA	poly(dimethylaminoethyl methacrylate)
PEG	poly(ethylene glycol)
PEGylation	conjugation of PEG to active agents
PEI	poly(ethylene imine)
PEtOx	poly(*N*-ethyl oxazoline)
PGlu	poly(glutamate)
PHPMA	poly(hydroxypropyl methacrylamide)
PIC	polyion complex
PLA	poly(lactide)
PLGA	poly(lactide-*co*-glycolide)
PLys	poly(lysine)
PMAA	poly(methacrylic acid)
PMVE	poly(methylvinylethers)
PNIPAAM	poly(*N*-isopropylacrylamide)
PNIPAM	poly(*N*-isopropyl acrylamide)
PPI	poly(propylene imine)
PPO	poly(propylene oxide)
PVP	poly(*N*-vinyl pyrrolidone)
RES	reticuloendothelial system
RNA	ribonucleic acid
siRNA	small interfering RNA
SLN	solid lipid nanoparticles

1 Introduction

1.1 Drug Delivery and Dendritic Drug Delivery Systems

1.1.1 Nanomedicine and Drug Delivery Systems

Nanomedicine can be described as the medical application of different life sciences, especially those with the focus on nanotechnology, to address diseases and dysfunctions of the human biological system. With the help of nanodevices and nanostructures, nanomedicine develops new and highly efficient methods to treat and cure these diseases.[1-5] New strategies like nanoformulations or nanocarriers have emerged from this field and are the modern approaches of drug delivery.

Drug delivery is the application of pharmaceutically active agents to the human body, including the administration and the localization at the target site until a therapeutic effect is achieved. The localization of a drug is fundamentally influenced by the way of administration. Nowadays, the following routes of administration are used: parenteral (intravascular, intramuscular, subcutaneous, and inhalation), enteral (oral, sublingual, and rectal), and topical (skin and mucosal membranes) (see Figure 1).[1, 6]

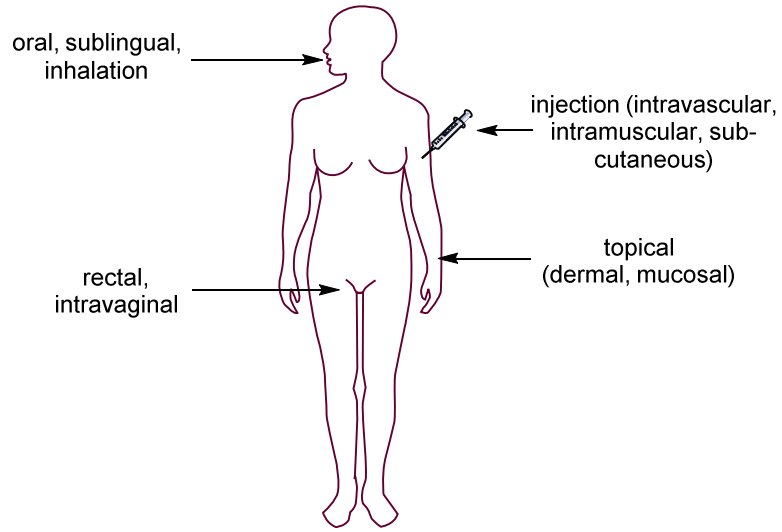

Figure 1. Main drug administration pathways: parenteral (intravascular, intramuscular, subcutaneous, and inhalation), enteral (oral, sublingual, and rectal), and topical (skin and mucosal membranes).

The fate of a drug administered by any of these ways finally depends on the physiochemical and biochemical properties of the drug and the biophysical properties of the body.[7] Since almost all drugs are not produced naturally in the human body, their pharmacokinetics is not optimized to achieve their highest pharmacological action. The majority of clinically used drugs are low-molecular-weight compounds, which have short half-life times in the blood stream and a high overall clearance rate (see Figure 2). They are distributed all-over the human body and diffuse easily into healthy tissues. The consequence is that only small amounts of the drug reach the target site. Therefore, high doses or repetitive injections are required to reach the therapeutic dose at the target site. Due to the lack of selectivity this leads to undesired side effects. Especially anti-cancer drugs can cause severe damages in healthy tissue if they do not reach their specific site of action as these types of drugs are highly cytotoxic.[7] However, not only toxicity is a problem in drug delivery.

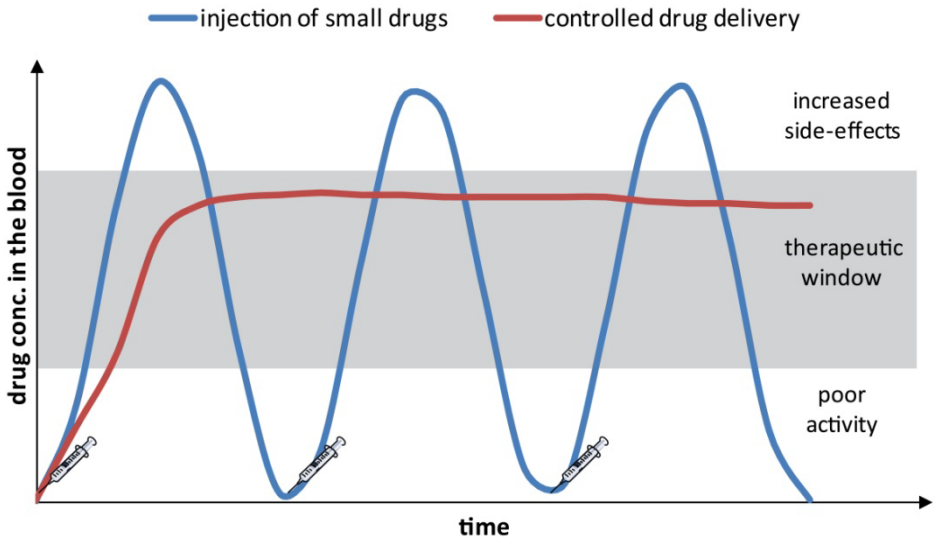

Figure 2. Comparison of drug concentration in the blood stream of a small-molecular-weight drug (blue curve) with a controlled drug delivery system showing idealized pharmacokinetics (red curve). While the small-molecular-weight drug needs to be administered several times, because it is rapidly cleared, the controlled drug delivery system remains at the desired concentration level within the therapeutic window of the drug.

Another one is the poor bioavailability of many potent drugs. Due to their often hydrophobic nature they cannot be administered and/or do not reach their site of action in the human body. Furthermore, evolution has perfected the defense mechanism of the human body against nano-

and micro-sized pathogens.[8] Biologically active agents like RNA and DNA, which are used in gene therapy, or proteins have the problem that they do not pass through cell walls, are often recognized as xenograft material, and are therefore rapidly degraded or excreted from the body by the immune system.[9-10]

To overcome these problems connected with the delivery of active agents different types of polymeric drug delivery systems (DDS) have been developed. They include polymers, which can act as active agents themselves, polymer conjugates, hydro- and nanogels, nanoparticles, as well as systems based on self-assembly approaches like polymeric micelles, polyplexes, and polymersomes (see Figure 3). The simplest approach to form a DDS is the conjugation of an active agent to high-molecular-weight polymers – resulting in so-called polymer conjugates. The most prominent polymer used for this purpose is poly(ethylene glycol) (PEG). The benefits of the conjugation of PEG (PEGylation) onto different active agents, like proteins, peptides, low-molecular-weight drugs and polynucleotides are manifold. PEG on its own has many beneficial properties, e.g., it is non-immunogenic, non-antigenic, and non-toxic as well as highly soluble in water and many organic solvents. Furthermore, it has been approved by the US Food and Drug Administration (FDA). PEGylation of a drug leads to reduced excretion by the kidneys, avoids or reduces degradation; it enhances the water solubility, reduces clearance by the mononuclear phagocyte system (MPS, formerly known as the reticuloendothelial system, RES), and reduces immunogenicity and antigenicity (mainly for peptides and proteins) of the conjugate.[11-12] The decreased interaction with blood components, e.g., complement activation and the achievement of long blood circulation times are known as “stealth” properties.[8, 13] Here, PEGylation is still considered as the gold standard to achieve the stealth effect. Due to the availability of only two (PEG) or even only one functional group in case of mono methyl ether poly(ethylene glycol) (mPEG), the amount of active agents that can be attached to PEG is strongly limited. Therefore, other concepts have been developed. For example, the attachment of several drug molecules via labile linker groups to a polymeric backbone which can carry additional solubilizing and targeting moieties as a general concept for a polymeric DDS was introduced by Helmut Ringsdorf in 1975.[14-15] Since then several polymer-based therapeutics have been developed and investigated.[16-17]

Further concepts involved the encapsulation of drugs into polymeric nanoparticles like hollow capsules of poly(lactide-*co*-glycolide) (PLGA).[18] Hydro- or nanogels consisting of cross-linked polymer networks have also been used successfully for the delivery of different drugs.[19] These types of DDS can be swollen and shrunken under certain conditions, allowing

the entrapment of drugs inside the gel. Later at the site of action, the gel is either degraded or swollen again to release the drug. Another DDS concept is based on the supramolecular self-assembly of surfactants or amphiphilic block copolymers. The thereby formed micelles and liposomes are inspired by their naturally occurring counterparts formed out of phospholipids, which are as well used for drug delivery. These polymeric micelles and polymersomes are widely and successfully used for the delivery of different active agents.[20-21] Polymeric micelles and polymersomes are more stable than their naturally occurring counterparts. Due to their bigger hydrophobic segments, the critical aggregation concentration (CAC) is

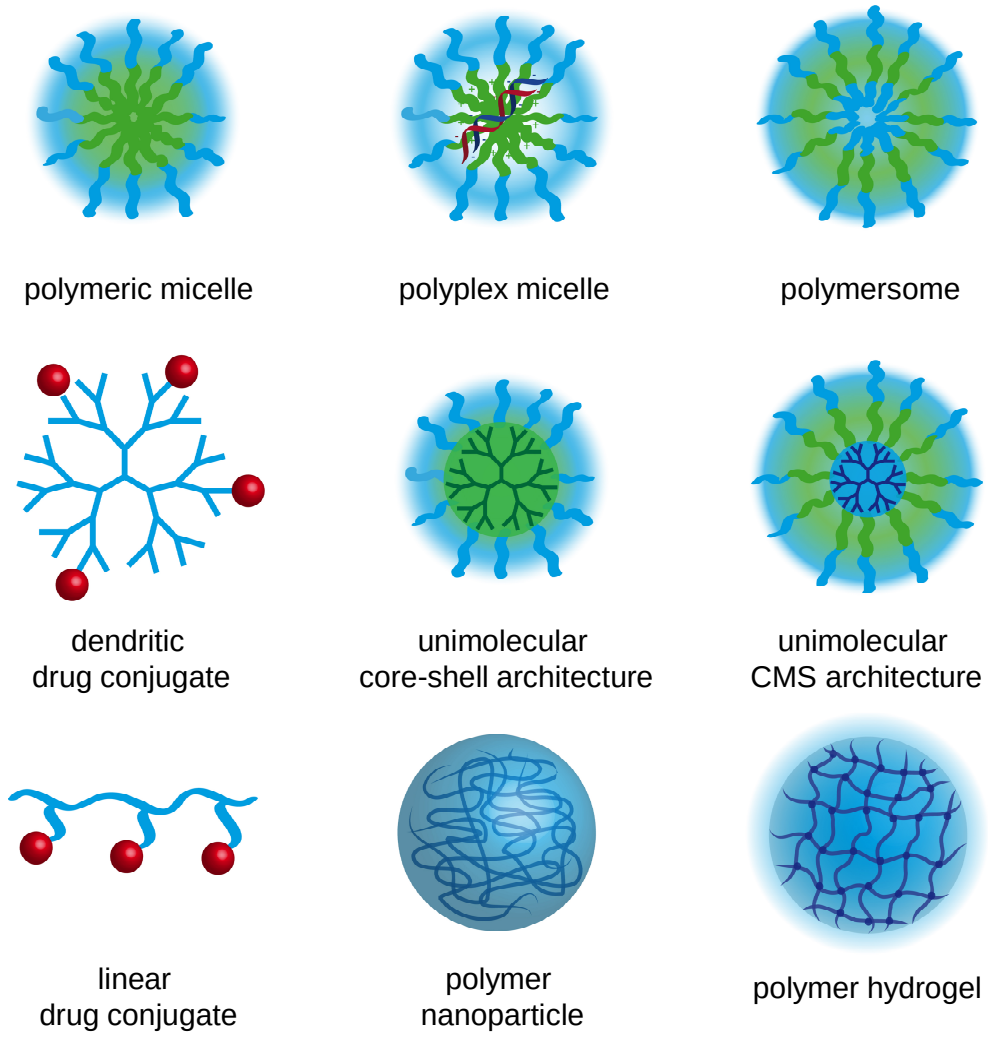

Figure 3. Overview of different polymer-based drug delivery systems: self-assembled polymeric micelles, polyplex micelles, and polymersomes; drug conjugates based on dendritic or linear polymer backbones; unimolecular particle based systems: core-shell and core-multishell (CMS) architectures, polymer nanoparticles and hydrogels.

significantly lower. Anyway, systems based on self-assembly can have stability issues upon application caused by dilution and interaction with blood constituents like proteins.[22] A similar but charge-driven process is used for the formation of so-called polyplex micelles. Here, a positively charged part of a block-copolymer is used to complex the negative charge of e.g. DNA.[20, 23]

The size of the DDS is also a very crucial factor which has to be considered for the successful delivery of active agents. Nanoparticulate systems have to tackle two major hurdles after systemic application, that are somewhat related to the size of the nanoparticle. First their renal excretion via the kidneys and second their capture by the MPS has to be circumvented. The threshold of renal clearance is reported to be around 45 kDa or between 4-9 nm in hydrodynamic diameter.[24-25] The MPS on the other hand is known to especially clear highly charged and larger particles from the human body, which have sizes bigger than several hundreds of nanometers. Regarding this, an ideal DDS that can achieve long systemic circulation has a size bigger than 10 nm, up to 200 nm, and is neutral in charge.[26-27]

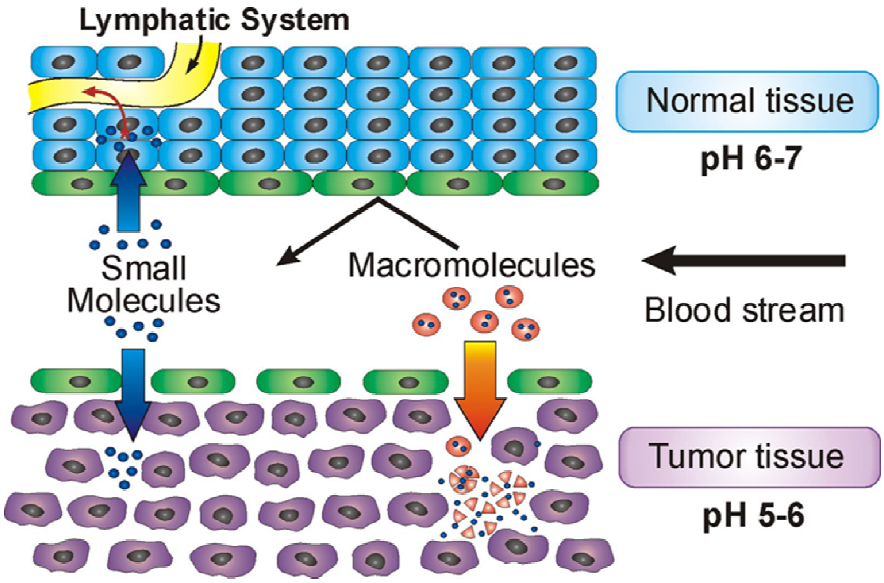

Figure 4. Schematic drawing of the permeability and retention properties of normal and tumor tissue in respect to small molecules and macromolecules (EPR effect). Figure reprinted from the literature.[16]

Only DDS that achieve long blood circulation can benefit from one of the major advantages of nanometer-sized polymeric DDS, which is the passive targeting that can be achieved by the enhanced permeation and retention (EPR) effect (see Figure 4).[28] Tumors

and inflamed tissue often have a more porous and disrupted endothelial cell layer due to their rapidly forming vasculature. Therefore, macromolecular architectures are able to penetrate into diseased tissue but are unable to cross the dense layer of endothelial cells present in healthy tissue. Small-molecular-weight drugs in contrast penetrate into diseased as well as healthy tissue which can lead to severe and undesired side-effects. In addition to the enhanced permeation into diseased tissue the less developed lymphatic drainage system causes an accumulation and retention of the macromolecules inside diseased tissue.

After the DDS has reached the diseased tissue it needs to cross the cell membrane in order to reach the drugs site of action. The plasma membrane regulates the entry and exit of all small and large molecules into the cytoplasm.[29] Many small molecules, like ions, sugars, and amino acids are brought into the cell by membrane protein pumps or channels. Larger particles, on the other hand, are transferred into the cell by endocytosis (see Figure 5). Among other properties, again the size of the DDS plays an important role. Particles with sizes > 1 µm are internalized by phagocytosis and those with smaller diameters by pinocytosis. The optimum size for cellular uptake of dendritic polyglycerol (dPG) without targeting ligands was found to be around 200 kDa/12 nm.[30]

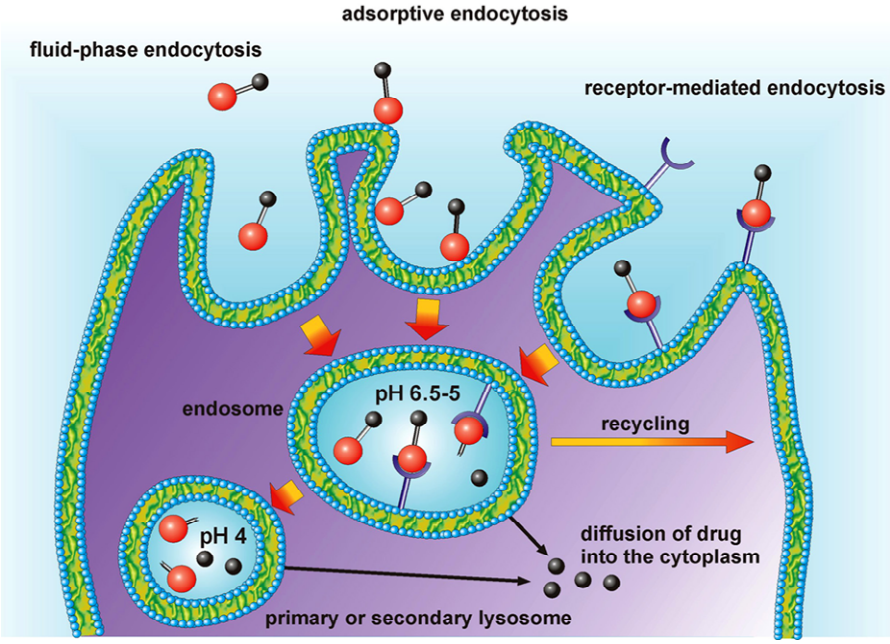

Figure 5. Schematic representation of the cellular uptake of macromolecules for drug delivery via endocytosis. Figure adapted from the literature.[16]

In general, all DDS should fulfill one or more of the following points in order to enhance the availability of the active agent:[31-32]

- Enhance the bioavailability of the applied drug by enhancing solubility, avoiding clearance, immune response and/or degradation
- Specifically transport the drug to the desired site of action, thereby avoiding its spreading throughout the whole body and reducing its systemic toxicity
- Release the drug in response to an external stimulus present at the site of action or locally applied from the outside of the body

Until now about a dozen nanomedicinal formulations that benefit from these advantages have been approved and are available on the market.[17] Eight of those are based on liposomes, for example Abelcet® (liposomal formulation of amphotericin B, anti-fungal drug) and the oldest example Doxil/Caelyx® (PEGylated liposomes of doxorubicin, anti-cancer drug). Abraxane® (albumin bound paclitaxel, anti-cancer drug), Adagen® (PEGylated enzyme, against severe combined immune deficiency), Oncaspar® (PEGylated adenosine deaminase, active against acute lymphoblastic leukemia) are further examples of marketed polymeric nanoparticles. Only one micellar nanomedicine, Genexol-PM® (PEG-poly(lactide) micelle formulation of paclitaxel, anticancer drug) reached the market, yet. Many more nanomedicines are under clinical evaluation. Altogether about 41 different nanocarriers are under investigation.[17] Additionally, one dendritic drug delivery system, Vivagel® (anionic G4-poly(L-lysine)-type dendrimer, against bacterial vaginosis) is in clinical trials and has already reached phase III studies.

1.1.2 Dendritic Drug Delivery Systems

Dendritic polymers evolved in the early 1980s. These macromolecules have a highly branched, tree-like architecture and were named dendritic polymers after the Greek word for tree ‘dendron’.[33-34] The class of dendritic polymers includes (i) dendrimers, (ii) dendrons, (ii) dendronized polymers, and (iv) hyperbranched polymers (see Figure 6). Dendrimers are perfectly branched architectures in the nanometer range. In case of perfect dendrimers, the polydispersity index (PDI) is one, meaning that the molecules are actually monodisperse. Dendrons, which are basically one branch of a dendrimer, also exhibit perfect structures. In

contrast, dendronized polymers and hyperbranched polymers are polydisperse architectures. Dendronized polymers usually have linear polymers as a backbone with dendrons or hyperbranched polymers attached to it. Hyperbranched polymers are similar to dendrimers but have higher PDIs (typically around 1.5-2) and are available in much bigger sizes (1-20 nm).

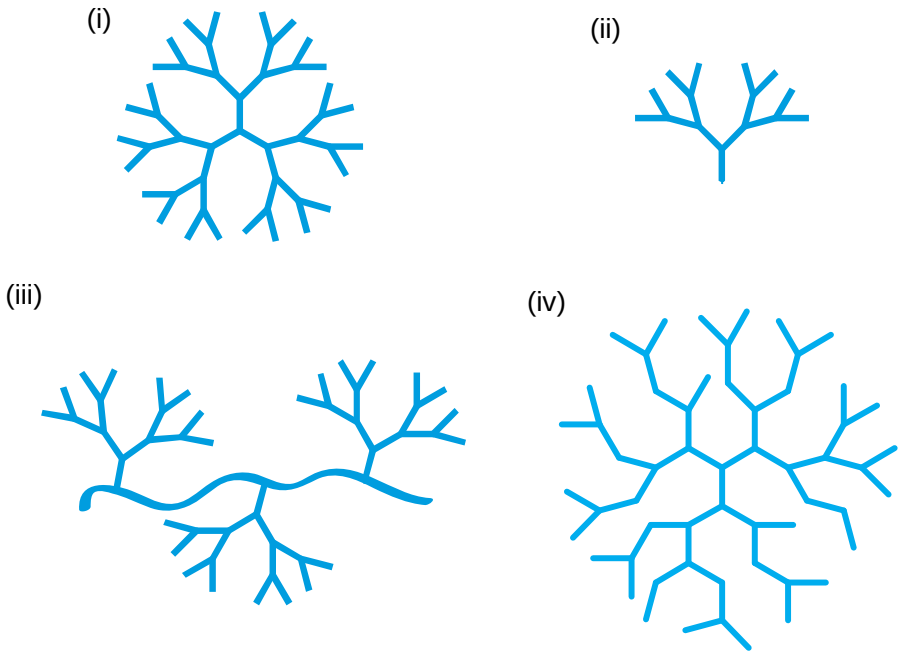

Figure 6. Schematic representation of the family of dendritic polymers: (i) dendrimers, (ii) dendrons, (iii) dendronized polymers, and (iv) hyperbranched polymers.

Common examples of dendritic polymers that have been widely used for biomedical applications are poly(propylene imine) (PPI), poly(amido amine) (PAMAM), poly(glycerol-co-succinate), and dendritic polyglycerol (dPG). Their structures are shown in Figure 7. Among all polymers used for the development of DDS especially dendrimers and hyperbranched polymers have properties that are highly advantageous for nanocarriers.[1, 32, 35-37] The advantages of dendritic polymers compared to similar linear polymers are their high number of functional groups which allow various modifications with functional moieties for solubilization, conjugation, targeting, and recognition, formation of internal cavities for encapsulation of guests, their high solubility[38-39] as well as a low intrinsic viscosity.[40-41] Furthermore, dendrimers show a strong ability to avoid the uptake by the non-specific MPS, they can easily passage across biological barriers by transcytosis, show a faster cellular entry, and their size in the nanometer range induces the passive targeting of tumor and inflamed

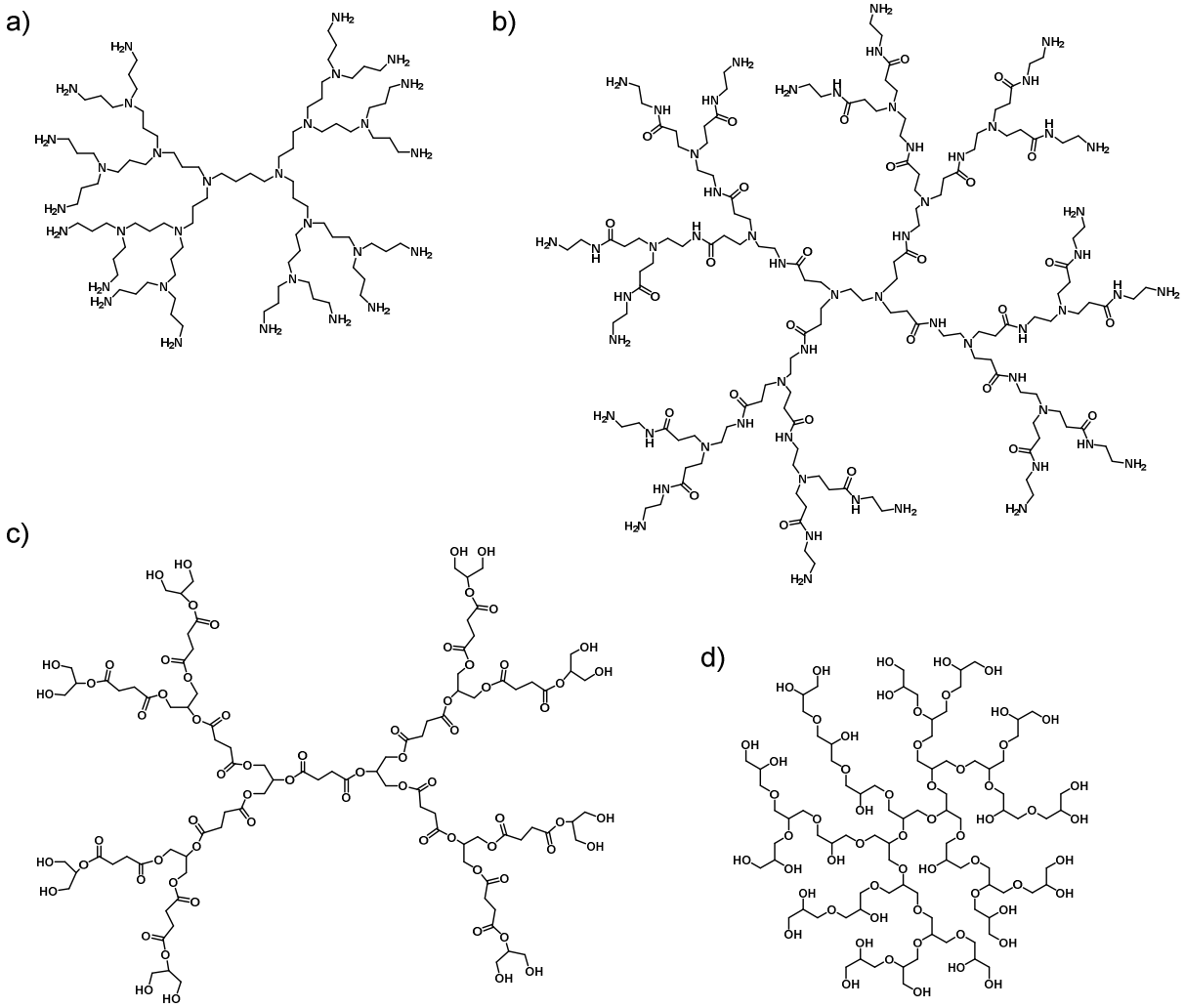

Figure 7. Examples of dendritic polymers that are commonly used as scaffolds for DDS: a) poly(propylene imine) (PPI), b) poly(amido amine) (PAMAM), c) poly-(glycerol-co-succinate), and d) dendritic polyglycerol (dPG).

tissue based on the EPR effect.[1, 32, 35] In addition, it was shown that with an increasing number of branches or arms the blood circulation half-life was increased compared to linear polymeric analogs with similar molecular weight and chemistry. This was shown with the help of several PEGylated polyester “bow-tie” dendrimers. For these bow-ties there was an 18-fold increase of the blood circulation half-life between the two-arm dendrimer (comparable to a linear polymer) and the four-arm dendrimer while retaining a similar molecular weight. The eight-arm dendrimer further led to a 1.2-fold increase of the blood circulation half-life. In vivo experiments showed no significant variation in tissue uptake between the polymers. Furthermore, a decreased polymer excretion via the urine with

increased branching was found. Overall, this dendritic DDS showed outstanding effectiveness in delivering doxorubicin to tumors of C26 colon tumor bearing mice.[42-43]

There are three principles of how to use dendritic polymers as DDS either by supramolecular complexation of the guest (Figure 8a), by covalent conjugation of the active species to the polymer (Figure 8b), or by using a dendritic polymer with a multivalent expression of pharmacologically active endgroups like sulfates and phosphates (Figure 8c).[44-46] In case of supramolecular complexes, the drug physically interacts with the dendritic backbone of the polymer by hydrogen bonding, hydrophobic interactions, or electrostatic interactions. The pioneering work of the Meijer group about the 'dendritic box' opened the way for the application of dendritic polymers as DDS. They utilized PPI dendrimers of generation five as carriers for Rose Bengal and *p*-nitrobenzoic acid and demonstrated the release of the guests upon a drop in pH.[47-48] As impressive as this initial work was, unfortunately the carrier was not water-soluble. However, inspired by this work many new dendritic polymers have been investigated for their suitability as DDS. To further enhance the transport capacities of the dendritic DDS they have been chemically modified. Most often the chemical modification is built up in such a manner that it forms a dense shell around the dendritic core which results in a barrier shielding the encapsulated guests against the surrounding medium. These so-called core-shell architectures will be discussed in the next subchapter (see Chapter 1.1.3). There have also been studies on unfunctionalized or only slightly modified dendritic polymers. Paleos et al. explored hyperbranched polyether polyols for the transport of pyrene and tamoxifen.[49] The complexation of PAMAM and a polyester dendrimer with ibuprofen has also been investigated. PAMAM dendrimers were able to complex more ibuprofen than the dendritic polyester, which was most likely due to the better interaction of the terminal amine groups of PAMAM with ibuprofen in contrast to the terminal hydroxyl groups of the polyester dendrimer. Interestingly, the complexes were stable in water and methanol and enhanced the cellular uptake of ibuprofen.[50] PAMAM was as well used for the transport of the highly efficient anti-cancer drug cisplatin which showed a higher in vivo activity if complexed by the dendrimer.[51] Dendritic polymers have been used for the formation of polymer-drug conjugates following the Ringsdorf model.[14-15] In this case drugs, targeting moieties and, if necessary, solubilizing agents were attached to the multiple functional groups of the dendritic molecule. In case of drug-conjugates the linker chemistry plays a key role, because the drug has to be attached in such a fashion that it remains active or it is released as its active form. Therefore, it is attractive to use linkages that can be cleaved under physiological conditions, e.g., esters, amides, or disulfides.

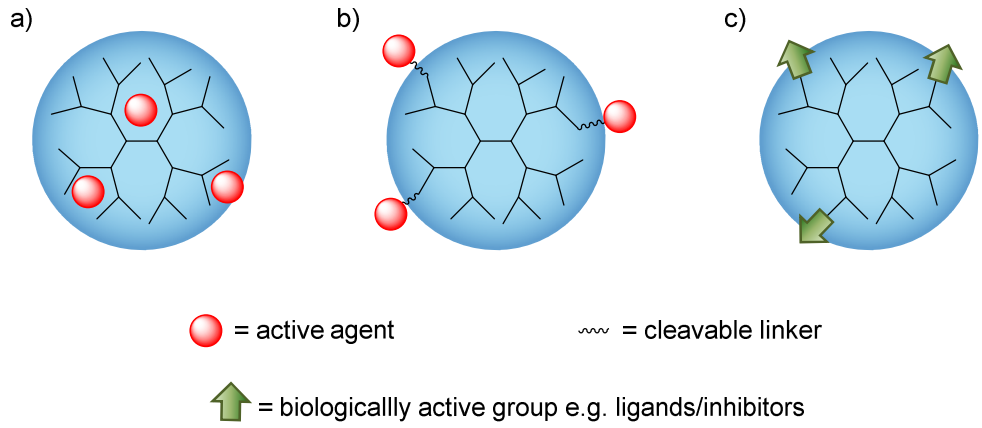

Figure 8. Three different principles of dendritic polymers as drug delivery systems: a) non-covalent/supramolecular encapsulation approach, b) dendritic polymer-drug conjugates, and c) multivalent presentation of biologically active groups, e.g., ligands or inhibitors on a dendritic scaffold. Figure adapted from the literature.[44]

An early example of a dendritic drug conjugate was introduced by Duncan and co-workers.[52] They used a PAMAM dendrimer with a carboxylate surface to covalently link cisplatin to the dendrimer, which could be released in vitro. Since then, a range of drugs has been conjugated to PAMAM derivatives, e.g., penicillin V,[53] the antidepressant venlafaxine,[54] 5-aminosalicylic acid,[55] and propranolol.[56] Other dendritic architectures have as well been used for the design of drug-conjugates. For example, the group of Fréchet synthesized dendritic polyesters and covalently attached the anticancer drug doxorubicin to these dendritic scaffolds.[57-58] The in vivo evaluation showed an increased serum half-life of the conjugate and less accumulation in the vital organs, proofing this conjugate to be a promising DDS.

Another application of positively charged dendritic architectures is gene delivery, which utilizes the strong interaction of the polyvalent charged scaffolds with the multiple negative charges of DNA/RNA strands. For example, guanidine-modified PPI showed excellent complexation of plasmid DNA and significant transfection efficiencies.[59] The Kissel group modified the commercial hyperbranched polymer Boltorn H with different amounts of surface amine functionalities and proved the ability to transfect cells based on the degree of amination.[60] Another successful approach to transfect DNA into cells based on spermidine-modified dendrons was chosen by Smith and coworkers.[61] Different dPG structures carrying spermine, spermidine, or pentaethylenehexamine groups were investigated in vitro with

regard to their gene silencing properties. The polyplexes yielded similar knockdown efficiencies and lower toxicity compared to the gold standard HiPerFect®.[62] Anionically charged dendritic structures also exhibit interesting properties as it was found that they possess anti-inflammatory properties.[63] Different dendritic scaffolds like PAMAM dendrimers terminated with carboxyl groups or aza bisphosphonate dendrimers have been used.[63-64] Sulfated dPG (dPGS) was successfully used to inhibit L- and P-selectin binding which plays a key role in inflammatory processes.[65] It was found that the size of the dendritic scaffold and the amount of sulfate functionalization were important factors for the binding affinity in this assay. Another important finding was that the actual functional group governing the anionic charge also plays an important role. By using sulfonate, carboxylate, phosphonate, and bisphosphonate groups on dPG scaffolds it was observed that sulfates are the most effective binders.[66-67] However, not only anti-inflammatory effects can be obtained with negatively charged dendritic architectures, e.g., dPG particles functionalized with sialic acid showed antiviral activity.[68]

1.1.3 Core-Shell and Core-Multishell Architectures for Drug Delivery

Compounds that exhibit a hydrophilic and a hydrophobic part, so-called amphiphiles, can undergo self-organization to higher ordered structures. The most prominent class of amphiphiles are phospholipids. Phospholipids consist of a diglyceride and a phosphate group. The diglyceride has two hydrophobic tails which are most often fatty acids, while the phosphate group functions as a hydrophilic head. Upon dilution in water the hydrophilic head group orientates towards the aqueous environment while the hydrophobic tails try to avoid the water phase and self-aggregate as a result of hydrophobic interactions. Their most important function is to form lipid bilayers building up cellular membranes. Besides the formation of cell membranes they are involved in the formation of vesicles which are important for several transport and regulation functions within living organisms, namely micelles and liposomes. Micelles are spherical, highly ordered self-assemblies with the hydrophilic part pointing towards the surrounding water. In this way an outer shell is formed, whilst the hydrophobic part points to the center of the micelle forming a hydrophobic core (see Figure 9a).[69] This is why micelles are considered to be core-shell architectures. The self-assembly process of micellar systems is controlled by the critical micelle concentration (CMC), which represents the minimum concentration of amphiphile which is necessary for the formation of micelles.

Liposomes are a second class of vesicles that can be formed by amphiphiles. They are composed of an aqueous core, encased by a hydrophobic bilayer forming an inner shell, and a hydrophilic outer shell which is in contact with the surrounding water (see Figure 9b).

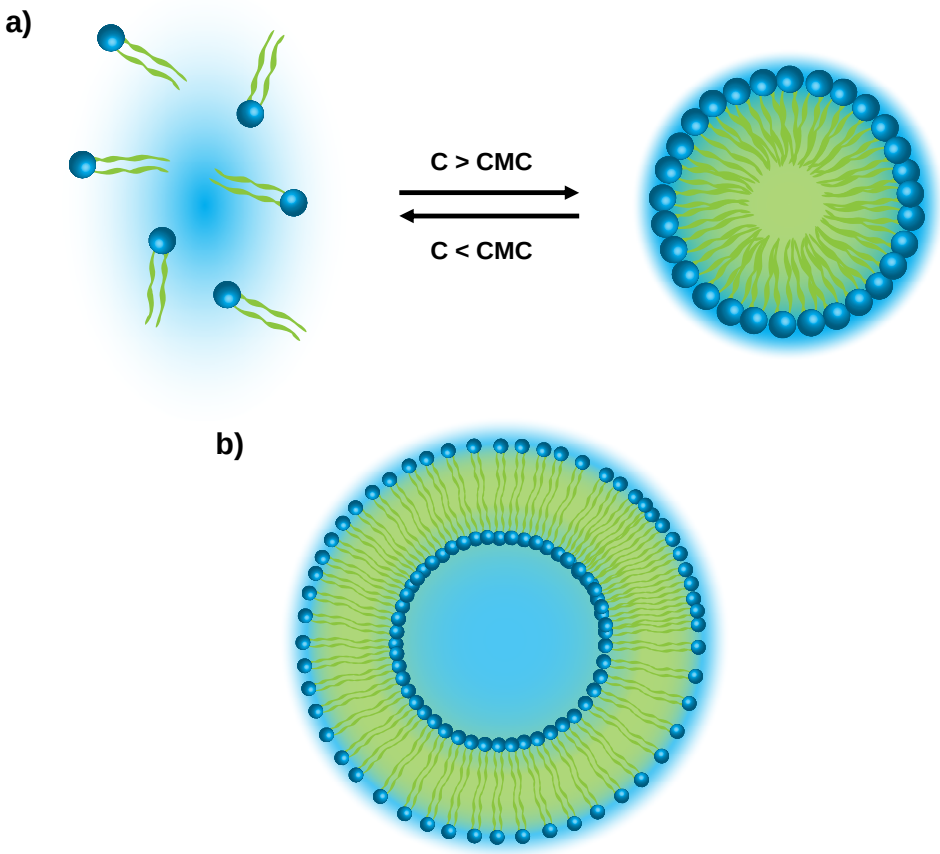

Figure 9. a) General scheme of the self-assembly process of a micelle. b) Bilayer structure of a liposome.

Inspired by naturally occurring transporters, polymeric core-shell and core-multishell architectures for drug delivery have been developed.[70] Polymeric micelles and polymersomes, are formed out of block-copolymers consisting of at least one hydrophilic and one hydrophobic block. Uncharged, hydrophobic drugs can easily be entrapped non-specifically in the hydrophobic core through hydrophobic interactions or by covalent attachment to the hydrophobic block. Charged hydrophilic guests like peptides, proteins, and nucleic acids can be incorporated in micelles having oppositely charged blocks to form so-called polyplexes or polyion complexes (PIC). Liposomes are able to entrap hydrophobic and hydrophilic drugs. Hydrophobic drugs are transported within the inner shell and hydrophilic drugs can be solubilized in the water which is entrapped in the core of the liposomal

architecture. Many different polymers can be used for the formation of the different segments. Polymers used for the hydrophobic block are for example PEG, poly(*N*-vinyl pyrrolidone) (PVP), poly(*N*-isopropyl acrylamide) (PNIPAM), and poly(hydroxypropyl methacrylamide) (PHPMA). Nevertheless, the most frequently used polymer for the hydrophilic block remains PEG,[20, 71] as it provides the above mentioned properties like stealth behavior to the micelle (see Chapter 1.1.1). Commonly used polymers for the hydrophobic block are for example poly(propylene oxide) (PPO),[72-73] poly(aspartate) (PAsp),[74-75] poly(lysine) (PLys),[76-77] poly(glutamate) (PGlu),[74, 78] poly(lactide) (PLA),[79-80] and poly(lactide-*co*-glycolide) (PLGA).[71, 81] Especially the degradable hydrophobic polymers derived from poly(amino acids), PLA, PLGA, and poly(ε-caprolactone) have been studied extensively. These polymeric micelles and polymersomes are used as DDS as they help to solubilize and protect the drug. Most importantly, the tuneability of the size in the nanometer scale enables passive targeting via the EPR effect. So far only one polymeric micelle (Genexol-PM®) came on the market in Korea and other Asian countries. It is based on a PEG-*b*-PLA block copolymer and is used for the delivery of paclitaxel. The same DDS reached phase III clinical trials in the US. Several other polymeric micelles using PEG-*b*-PAsp, PEG-*b*-PGlu diblock polymers or PEG-*b*-PPO-*b*-PEG triblock polymers have reached phase I-III clinical trials in different countries.[17] In the case of liposomal architectures so far only liposomes using non-polymeric amphiphiles have found market approval or reached clinical trials. The only liposome involving a polymer that got approved is Doxil®/Caelyx® based on PEGylated lipids.[17] A further benefit of these synthetic vesicles is that they can be designed in such a way that one can attach additional targeting moieties on the surface to achieve active targeting.

Though the term active targeting is frequently used, it is slightly misleading as the attachment of targeting ligands does not change the route of the DDS in the body. In any case, it first has to reach its site of action by the same route as a non-targeted DDS. The benefit is the increased retention of the DDS in a certain tissue due to the interaction of the targeting ligands with specific receptors that can be overexpressed in the target tissue. Furthermore, the cellular uptake can be enhanced due to receptor-mediated endocytosis. Commonly used targeting ligands are monoclonal antibodies, glycoproteins, lipoproteins, carbohydrates, folic acid, human transferrin, or peptides. For example Yoo et al. successfully attached folate groups to PEG-*b*-PLGA micelles and were able to enhance the cellular uptake compared to micelles bearing no folate groups.[81] Increased transfection efficiency was reported by Oba et al. for plasmid DNA-loaded PEG-*b*-PLys micelles having a cyclic RGD peptide (cRGD) on the micelle surface compared to PEG-*b*-PLys micelles without cRGD.[77] The enhanced

transfection efficiency was only observed for HeLa cells which overexpress the corresponding receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors) for cRGD and not for 293T cells where no overexpression was observed. A very recent example using cRGD-functionalized (1,2-diaminocyclohexane)platinum-loaded PEG-*b*-PGlu micelles to target human glioblastoma brain tumors was published by Miura et al.[82] Here, the cRGD enabled transcytosis across the well defined vascular barriers found in the brain (e.g. blood brain barrier) that make the targeting of this type of tumors exceptional difficult. These findings indicate that cRGD-mediated DDS are a powerful tool for treating glioblastoma.

A further big advantage of the polymeric micelles is their lower CMC or in case of polymeric systems also often called CAC. Micelles easily fall apart if they are diluted below their CMC. The increased stability of polymeric micelles in case of dilution derives from the larger hydrophobic parts, since it is much less favorable to have large hydrophobic domains exposed to the surrounding water. However, many factors that possibly influence the stability of polymeric micelles are not yet fully understood and sufficiently investigated. Almost all studies focus on water or aqueous buffers as media to investigate micelle formation and destabilization but leave out physiologically relevant media, like human blood or serum.[22] In order to further increase the stability of polymeric micelles, different approaches have been used. One method is the cross-linking of the core or the corona of a micelle after its formation. The group of Kataoka developed a micelle bearing iminothiolane units in the hydrophobic domain.[83] After self-assembly, the thiols of the iminothiolane units were oxidized to form disulfide bridges between the polymer chains preventing the dissociation of the single amphiphiles. Additionally, this modification renders the resulting micelles stimuli-responsive to the reductive potential of the surrounding environment, e.g. an increased concentration of glutathione (GSH) in the cytoplasm (see Chapter 1.2). With this approach PIC micelles loaded with siRNA achieved a 100-fold enhancement of the transfection efficiency.[84] On the other hand, in case of too high cross-linking a significant decrease of the transfection efficiency was observed due to over-stabilization.[85]

Another approach towards stable core-shell architectures are the so-called unimolecular micelles which consist of a hydrophobic dendrimer or hyperbranched polymer functioning as a core and a covalently attached dense polar shell. Unlike micelles, these nanocarriers can not fall apart upon dilution. The term unimolecular micelle was introduced by Newkome et al. with their report about [27]-arborol.[86] Since then many examples of unimolecular micelles have been reported. For example, by functionalizing PPI with 3,4,5-tris-(tetraethyleneoxy)benzoyl groups a water soluble unimolecular micelle was obtained, that

transported Rose Bengal and 4,5,6,7-tetrachlorofluorescein in its core.[87] Kojima et al. used PEG-grafted PAMAM dendrimers to encapsulate the drugs doxorubicin and methotrexate.[88] They were able to show that the amount of drug increased with increasing core and shell size. Fréchet and co-workers also grafted PEG to their dendrimer derived from 4,4-bis(4′-hydroxyphenyl) pentanol monomer units and could successfully encapsulate pyrene and indomethacin.[89] Using a different approach, our group synthesized unimolecular core-shell architectures upon modification of the inner part of dPG with hydrophobic groups.[90-91] Many more unimolecular core-shell architectures have recently been investigated. Most of them are designed to be responsive to certain stimuli to facilitate the release of the encapsulated guest molecules and therefore will be discussed in Chapter 1.2.2.

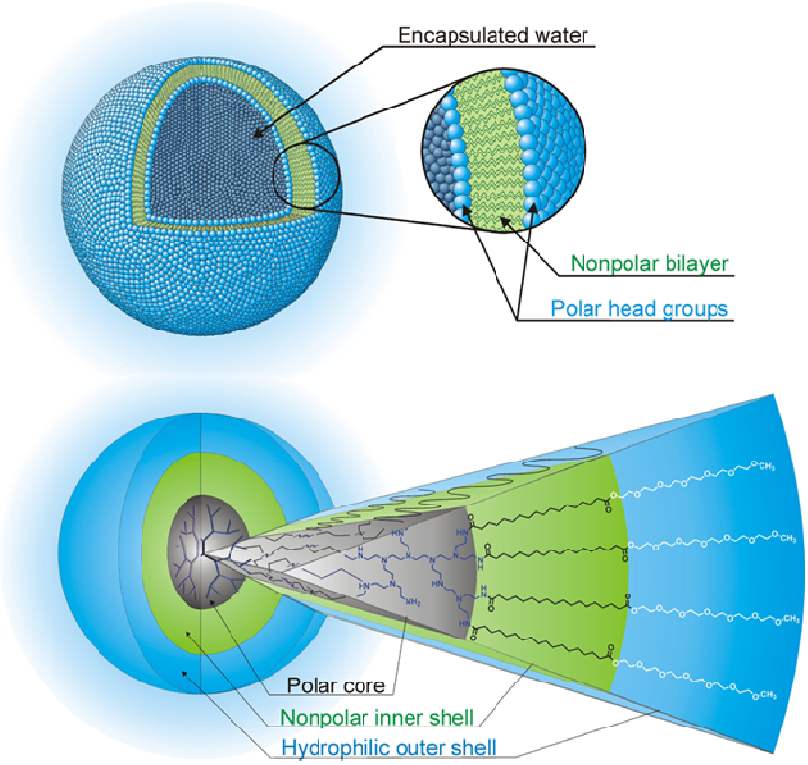

Figure 10. Schematic representation of a liposome (top) and a unimolecular core-multishell (CMS) architecture (bottom). Figure reprinted from the literature.[92]

By adding at least one additional shell around the dendritic core one ends up with core-multishell (CMS) architectures. The extra shell can contribute with many additional functions allowing for a vast range of particles with numerous properties. A polymersome-based approach towards a CMS DDS was conducted by using a double emulsion process with a

mPEG-*b*-PGlu block copolymer.[93] Due to the CMS architecture these polymersomes were able to encapsulate a hydrophobic as well as a hydrophilic anticancer drug achieving a synergistic effect. Unimolecular CMS nanocarriers for example have been formed by attaching mPEG-*b*-PLA or mPEG-*b*-PGlu block copolymers to Boltorn or hyperbranched poly(ethylene imine) (PEI).[94-95] Our group established a novel and highly versatile type of unimolecular CMS architectures initially based on hyperbranched PEI and later on dPG.[96] These unimolecular CMS architectures were synthesized by attaching long bifunctional alkyl chains with mPEG attached on one end, to the dendritic cores. The design of these CMS architectures was inspired by the polarity variation of liposomes, having a polar core with a hydrophobic inner layer and a polar exterior (see

Figure 10). CMS nanocarriers are soluble in water and in most organic solvents and they are able to solubilize hydrophobic as well as hydrophilic guests. Therefore, these universal nanocarriers are highly suitable as DDS. Surprisingly, the transport of guest molecules did not occur via unimolecular particles as originally intended but instead via the formation of supramolecular aggregates of the nanocarriers. This aggregation behavior was not observed when the outer shell was exchanged by a grafted hyperbranched PG shell.[97] The CMS nanocarriers found various biomedical applications like in vivo targeting of a F9 teratocarcinoma tumor and the modulation of the copper level in eukaryotic cells.[92, 98] It was shown that CMS nanocarriers benefit from the EPR effect and are therefore able to deliver their payload more selectively into tumor tissue (see Figure 11).[92] Furthermore, it was found that CMS nanoparticles are capable of enhancing the skin penetration of different guest molecules, which will be discussed in more detail in Chapter 1.3.[99-100] Besides their biomedical applications these CMS nanocarriers were shown to stabilize different metal nanoparticles like gold, platinum, and palladium which subsequently have been used for

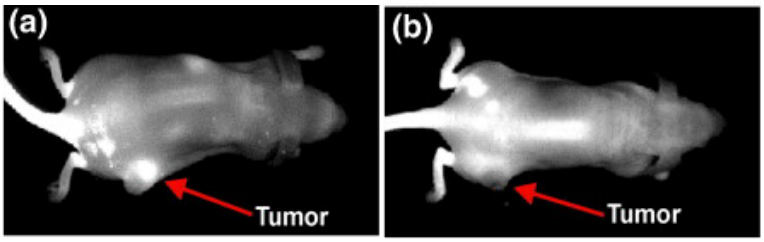

Figure 11. Laser diode/camera assembly image of F9 teratocarcinoma bearing mice: (a) Strong contrast was observed after 6 h of administration of ITCC dye-loaded CMS nanocarriers to F9 teratocarcinoma bearing mice; (b) the contrast achieved with free dye after the same time period is not very prominent. Figure adapted from the literature.[92]

different catalytic reactions.[101-104] In a similar approach the CMS nanocarriers were used as templates for the formation of platinum nanoparticles in mesoporous silica.[105] Although these CMS nanocarriers have already proven to be highly versatile and suitable for biomedical applications, so far they have not been designed in such a fashion that their payload can be released upon action of an external trigger.

1.2 Stimuli-Responsive Nanocarriers

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1.2.1 Stimuli for Triggering Drug Release

The advantages of polymeric DDS are manifold as described above (see Chapter 1.1.1). Additionally, the progress in chemistry and materials science allows the tailoring of DDS that can selectively release their cargo at the desired site of action and in response to an external stimulus (see Figure 12). In order to trigger the release, these responsive DDS are designed to react to certain stimuli like pH, temperature, redox potential, enzymes, light, and ultrasound.

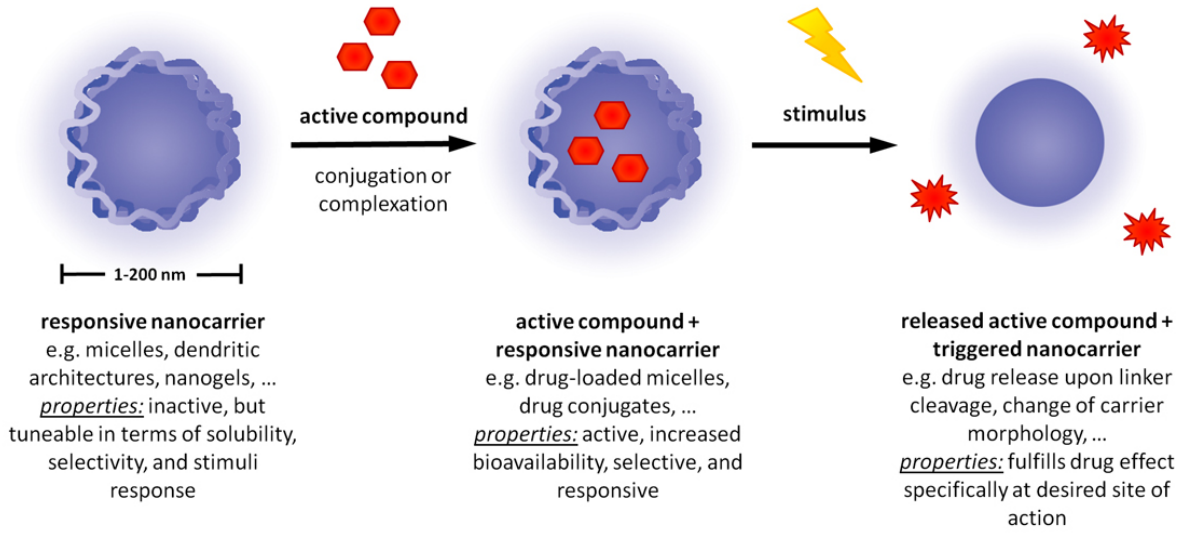


Figure 12. General scheme of a stimuli-responsive nanocarrier for the transport of active compounds. Figure reprinted from the literature.[106]

The central operating principle of responsive DDS is based on specific cellular/extracellular stimuli of chemical, biochemical, or physical origin that can change the structural composition/conformation of the nanocarriers, thereby promoting the release of transported guests. The changes are mainly decomposition, ionization, isomerization, depolymerization, or activation of supramolecular disaggregation among many others. The general concept of triggered release, as shown in Figure 13, can be divided into two major modes according to the design of the nanocarriers. In the complexation approach (Figure 13a),

where the bioactive agent is entrapped within the nanocarrier, the release can be triggered by structural changes within the carrier scaffold (e.g., carrier degradation, cleavage of shell, charging of functional groups), while in the nanocarrier-conjugate approach, the release involves cleavage of the linker between carrier and bioactive agent (Figure 13b). Thus, these advanced nanocarriers become rather an active participant in the therapeutic landscape than only being an inert carrier molecule.[107]

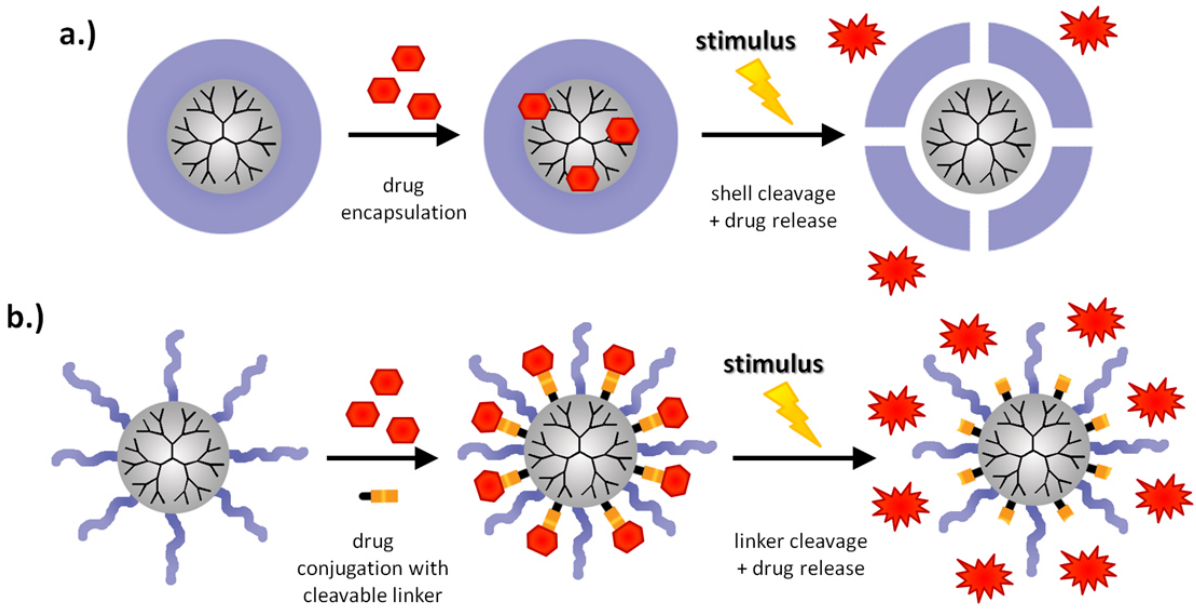


Figure 13. Different mechanisms for stimuli-responsive release of active agents from nanocarriers: a) supramolecular complexes like dendritic core-shell particles with a cleavable shell and b) dendritic scaffolds with attached solubilizing/stealth groups using cleavable linkers for the drug conjugation. Figure reprinted from the literature.[106]

The benefits of responsive DDS are essentially important when the stimuli to which they act are disease or systemic-biochemistry specific (e.g., to a defined enzyme class, specific protein overexpression, pH, electrolyte status). Such specificity allows the nanocarriers to precisely release their cargo in a temporal or spatial pattern in response to particular pathological triggers present in the diseased tissues resulting in substantially reduced side effects.

Chemical and biochemical stimuli that can be used to trigger the release from a DDS include cellular pH-shifts, changes in the redox and ionic microenvironment of the specific tissues, enzyme overexpression in certain pathological states, host–guest recognition, and antigen–antibody interactions.[108] In many diseases the normal pH-gradient between extra-

and intracellular environment is greatly affected. For example, in solid tumors, the extracellular tissue can be more acidic (~ 6-7) than the systemic pH (7.4) due to poor vasculature and consequent anaerobic conditions.[109] The cellular organelles also exhibit sharp pH differences in different locations, for instance, in cytosolic, endosomal, and lysosomal compartments. A pH-responsive DDS can respond to such pH-differences and release the payload at the site of action either by destabilization of the whole nanocarrier or by cleavage of pH-sensitive linkages that connect the drug molecules to the carrier. pH-sensitive moieties that can be incorporated to achieve structural changes of the carrier include carboxyl and/or tertiary amines. These functional groups alter their hydrophobicity/hydrophilicity upon protonation/deprotonation which can be used to destabilize the nanocarrier.[110-115] The most commonly used polymers for this purpose are poly(acrylamide) (PAAm), poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), poly(diethylaminoethyl methacrylate) (PDEAEMA), and poly(dimethylaminoethyl methacrylate) (PDMAEMA).[113-114] In order to conjugate drugs to nanocarriers or to make a polymer backbone pH-cleavable, linkages such as hydrazone, hydrazide, and acetals are used (see Figure 14).

Another biochemical property, which is used to trigger the release from responsive DDS, is the presence of enzymes, such as proteases, glucuronidase, or carboxylesterases, which are often overexpressed by malignant cells and are either intra- or extracellularly presented. The protease cathepsin B, that degrades proteins in lysosomes, has been intensively investigated for the development of enzyme-responsive DDS. In general, proteases that are extracellularly expressed, such as the matrix metalloproteases, are specific biomarkers of malignant tissues. They are responsible for the proteolysis of the extracellular matrix and basement membranes and are required during embryo morphogenesis, tissue remodeling, angiogenesis, and parasitic or bacterial invasion.[116-119] The drug release can be achieved by introducing specific enzyme substrate sequences either into the nanocarrier scaffold, or into the linker segment through which the drug is anchored to the nanocarrier. These enzyme-cleavable groups can range from simple ester and carbamate linkers, that are hydrolyzed by proteases (see Figure 14), or amino acid sequences that are explicit substrates for certain enzymes. Tumor tissues are enriched with proangiogenic and angiogenic enzymes. Most importantly, such hypoxic areas are environmentally reductive due to the presence of bio-reductive enzymes.[120-123] In normal tissue, the intracellular GSH level ranges from 1 to 10 mM compared to that of blood plasma which is 2 μM.[120, 124-125] In vitro the GSH levels were found to be 7-10-fold higher in tumor cells than in normal cells. This combination of intracellular elevated GSH and the tumor-associated GSH make redox-responsive nanocarriers interesting candidates for targeted drug

release.[120] The DDS in this case contains a disulfide group to either conjugate the drug or cross-link the nanocarriers. The disulfide bond is broken down by GSH into two thiol moieties releasing the drug.[126]

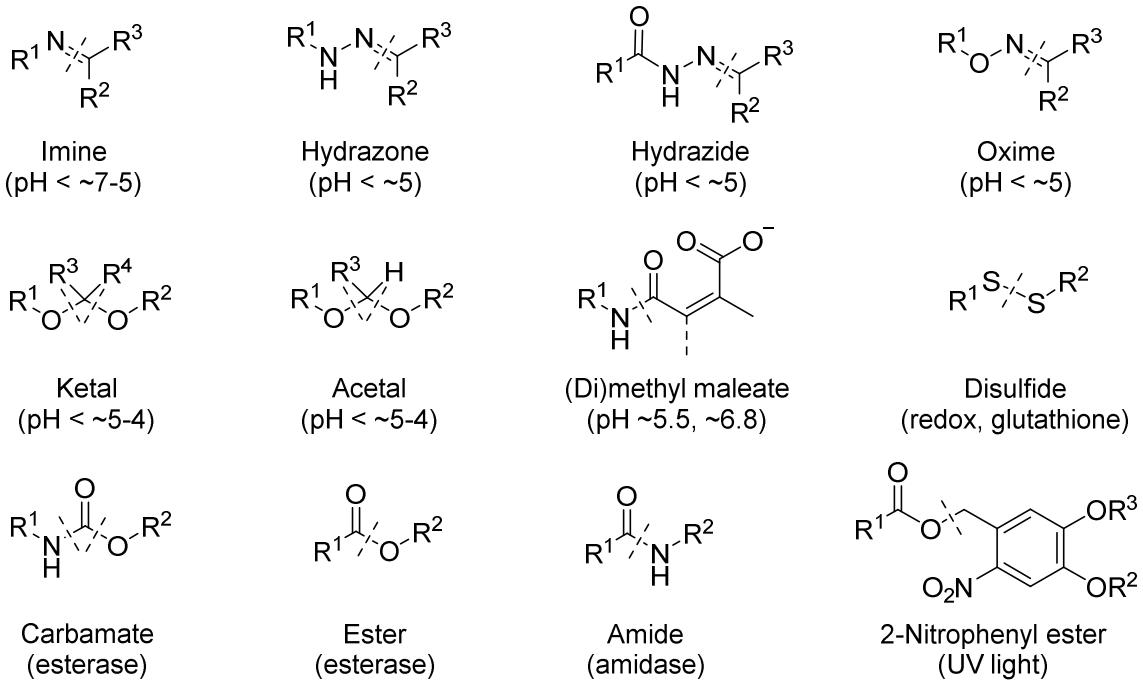


Figure 14. Cleavable linkers used for stimuli-responsive nanocarriers. The dashed line shows the bond that is broken upon activation by the corresponding stimulus which is given in parentheses. Figure adapted from the literature.[106]

Physical stimuli that are used for the triggered release of drugs involve temperature, light, and strength of magnetic or electrical fields.[127] In thermo-responsive nanocarriers, a temperature-sensitive polymer is used, which allows the delivery system to release the payload upon changes in temperatures. The drug release can only be achieved after subsequent application of differential temperature, for instance, in the form of hyperthermic stimuli at the target tissue. For this purpose, polymers with a lower critical solution temperature (LCST) are used. If the polymer faces an environment that is heated above the LCST the polymer backbone is dehydrated and the polymer becomes more hydrophobic. This change in the hydrophilic-hydrophobic balance causes a destabilization of the DDS and leads to a release of the drug. Polymers that have been used for this purpose are poly(*N*-isopropylacrylamide) (PNIPAAM), poly(*N*,*N*-diethylacrylamide (PDEAM), poly(methyl-

vinylethers) (PMVE), and poly(*N*-ethyl oxazoline) (PEtOx).[113-114] Depending on their molecular weight, PEGs also exhibit a LCST. Usually the LCST of PEG is too high which can be overcome by copolymerization with other monomers. Feasibility of local/regional heat deposition and hyperthermia induced vascular permeability additionally endows the thermo-responsive nanocarrier with the advantage of remote targeting in passive mode.[120] The use of light as an external stimulus offers a range of advantages, including ease of application, relative biocompatibility and controllability both spatially and temporally.[128-132] The principle of photo-responsive dendritic architectures relies on the adjustable release of encapsulated/conjugated bioactive units from the structure under the influence of light of a specific frequency.[108] In particular, radiation of UV, near IR, and IR frequency are generally used which are tissue compatible, yet powerful enough to bring about conformational changes within the nanocarriers' chemical architecture, e.g., the cleavage of a 2-nitrophenyl ester linkage (see Figure 14). Ultrasound attracted growing attraction for targeted and responsive DDS. Ultrasound can be used as a trigger to release active molecules from polymeric matrices by regional sonication. Ultrasonic-mediated contrast agent release from nanocarriers with the aid of microbubbles is a well-established technique in the field of diagnosis. In addition to tumor uptake, this technique also allows the uniform distribution of micelles and drugs throughout the tumor tissue.[133]

1.2.2 Stimuli-Responsive Core-Shell and Core-Multishell Architectures

The utilization of biochemical stimuli that occur within the body is of particular interest as it supersedes the application of additional external triggers. Therefore, this chapter will focus on pH- and redox-responsive as well as enzymatically degradable DDS. Here, pH-responsiveness plays an important role in controlled release as sharp pH gradients exist in some diseased tissues, like tumor and inflamed tissue. Furthermore, the pH of different cellular compartments during endocytosis also differs significantly from the physiological pH allowing for intracellular release. Core-shell DDS used for this purpose include block copolymer micelles consisting of poly(histidine)-*b*-PEG and PLA-*b*-PEG-*b*-poly(histidine).[134-135] Upon a pH drop, the hydrophobic part is protonated which changes it into a hydrophilic block causing the disassembly of the micelle. The Kataoka group attached the anticancer drug doxorubicin via a pH-labile hydrazone linker to a PEG-*b*-PAsp block copolymer micelle, thereby significantly enhancing the drug release at pH 6 or lower.[136-137]

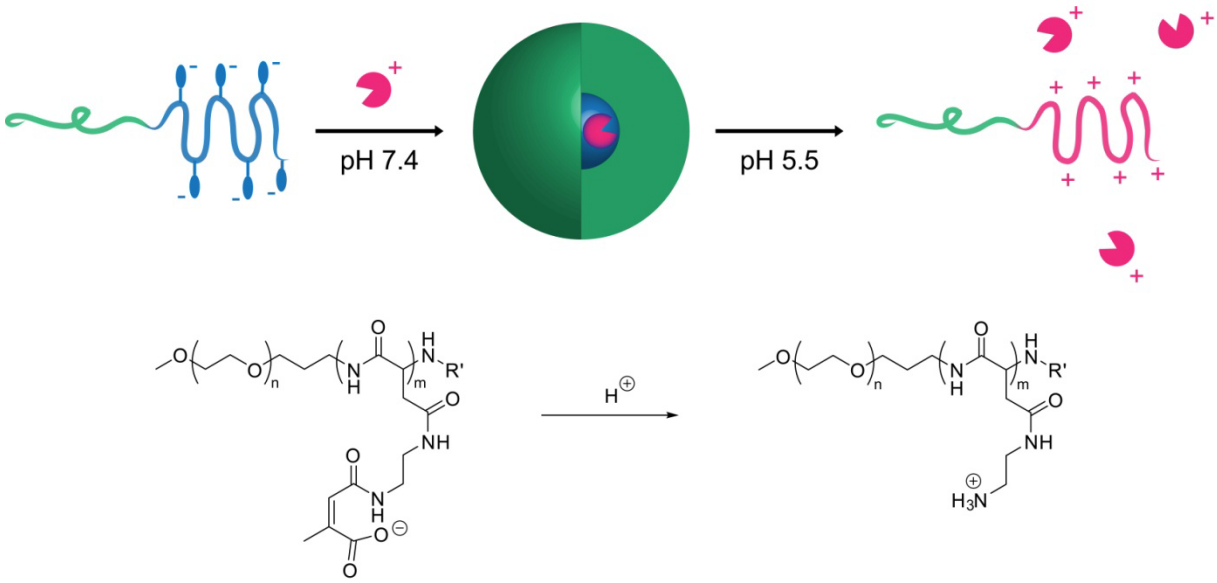


Figure 15. Formation and dissociation of a charge-conversion micelle encapsulating and releasing a positively charged protein in dependence on the polymer charge. Figure adapted from the literature.[138]

Furthermore, the same group synthesized charge-conversion micelles for the delivery of positively charged proteins by attaching methyl maleate groups to the aspartate block.[138] The micelles were negatively charged and stable at physiological conditions. At lower pH, the methyl maleate groups were cleaved, resulting in a positively charged aspartate block which led to the disassembly of the micelle and release of the protein (see Figure 15). Our group established several unimolecular pH-responsive core-shell architectures based on hyper-branched PEI and dPG. The attachment of alkyl chains via acetal, ketal, or imine groups resulted in inversed unimolecular micelles,[139] while the attachment of PEG shells via an imine bond resulted in water-soluble core-shell nanocarriers.[140-143] These architectures could be cleaved at pH values between 5 and 7 depending on the pH-labile group. The cleavage resulted in the release of various guest molecules that had been encapsulated beforehand. Following the drug-conjugate principle, Haag and co-workers also used the hydrazone linkage to attach doxorubicin in addition to a PEG shell to dPG. By using this conjugate, complete tumor remission for 30 days was achieved in mice. Even though three times the maximal tolerated dose compared to free doxorubicin was administered as drug conjugate, no significant loss of body weight of the mice was observed.[144] A DDS based on PAMAM with an DMAEMA-*b*-PEG double shell was described by Shen et al. that released the encapsulated

anticancer drug paclitaxel upon protonation of the DMAEMA block causing the inner shell to stretch out.[145]

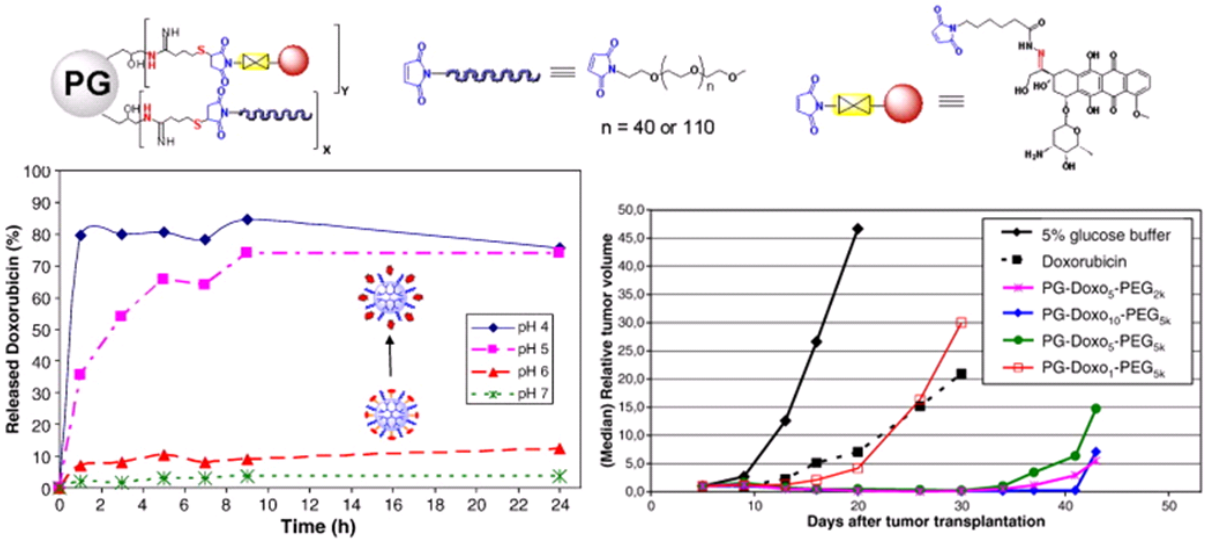


Figure 16. Schematic structure of doxorubicin–polyglycerol conjugates. The curves depict the pH-release profile and the tumor growth inhibition of subcutaneously growing A2780 xenografts under therapy with doxorubicin and the conjugates. Figure reprinted from the literature.[144]

Another stimulus used as trigger is the oxidative or reductive nature of certain environments. In general, the extracellular space is oxidative while the intracellular space is reductive, which is strongly related to the GSH concentration in the different environments (see Figure 17).[146] Therefore, redox-sensitive DDS are of particular interest for intracellular delivery, e.g., in gene delivery, where it is crucial to protect the plasmid DNA or siRNA until it reaches the cell interior. As already mentioned in Chapter 1.2.1, the cross-linking of thiol-containing blocks from block copolymer micelles is a promising approach for the stabilization of micelles as well as the introduction of responsiveness. The Kataoka group achieved a 100-fold increased siRNA-transfection by using cross-linked PIC micelles.[84] Recently, they have used the same micelles to encapsulate the photosensitizer phthalocyanine and were able to show enhanced phototoxicity.[148] A cleavable disulfide bond can also be incorporated into the amphiphile structure, thereby linking the hydrophilic with the hydrophobic block. Amongst others, Wang et al. demonstrated this by attaching a poly(ε-caprolactone) block to a poly(ethyl ethylene phosphate) block.[149] The same approach was used to attach a shell of spermidine moieties to a dendritic polymer in order to complex and release DNA. The

resulting complexes were rapidly cleaved in a reductive environment, proving that single spermidine molecules are not able to complex DNA.[150] Kono and coworkers used cystein functionalized PAMAM dendrimers that were further coated with a PEG shell to confine the guest molecules upon cross-linking of the thiol groups of cystein.[151]

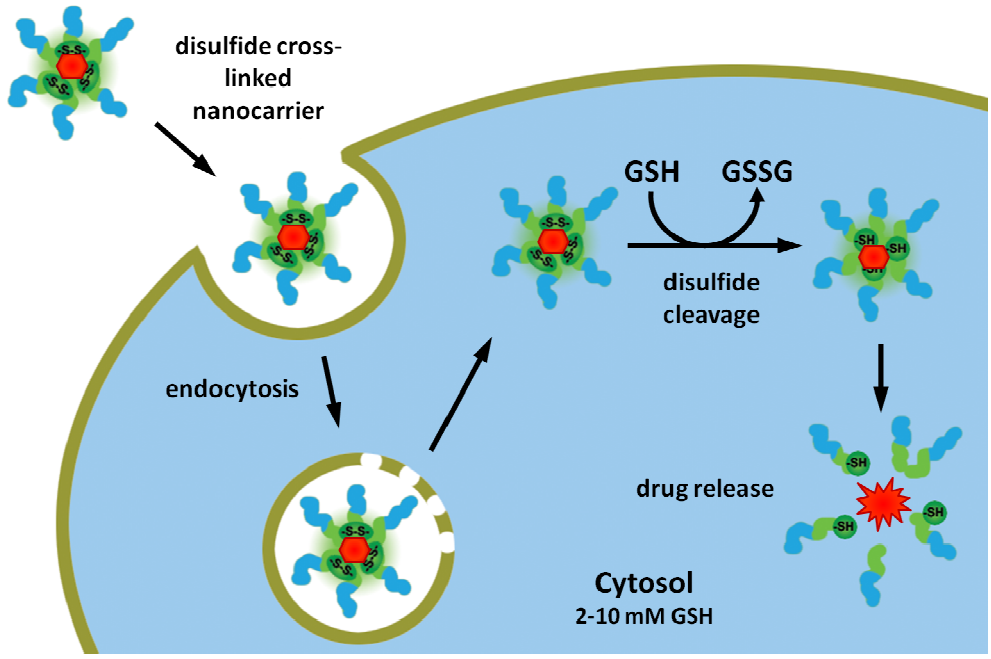


Figure 17. Schematic representation of the mechanism of action of redox-responsive nanocarriers. The drug loaded carrier is taken up into the cell by endocytosis. After reaching the cytosol the disulfide bonds are reduced by glutathione (GSH) and the carrier falls apart releasing the drug into the cytosol. Figure reprinted from the literature.[106]

Nowadays, there have been several attempts to design dual or even multi-responsive DDS.[152] Especially the combination of pH- and redox-response is of particular interest as both stimuli are active in certain pathological sites as well as in all cells. Shuai et al. synthesized such a dual responsive DDS based on PEG-*b*-poly(aspartic acid/ mercaptoethylamine)-*b*-poly(aspartic acid/2-(diisopropylamino)ethylamine (PEG-*b*-PAsp-(MEA)-*b*-PAsp(DIP)).[153] The triblock polymers form micelles at pH 10 and were subsequently stabilized by oxidative cross-linking and it was possible to release the loaded doxorubicin at pH 5 or under reductive conditions. The release was further enhanced if a reductive environment at pH 5 was chosen. In vivo studies showed reduced drug leakage in the blood stream and an increased therapeutic effect. Another very recent example of a highly effective dual responsive micelle was reported by the group of Zhong.[147] They used a PEG-

b-PLys block copolymer and partially modified the PLys segment with lipoic acid and *cis*-1,2-cyclohexanedicarboxylic acid (see Figure 18). The lipoic acid was used to cross-link the micelles after self-assembly making the micelle redox-responsive and the *cis*-1,2-cyclohexanedicarboxylic acid was applied to obtain the pH-responsivness, as it undergoes a charge conversion at low pH from negatively to positively charged. Under the combination of acidic and reductive conditions the micelles released more than 95 % of the loaded doxorubicin resulting in significantly pronounced cytotoxic effects.

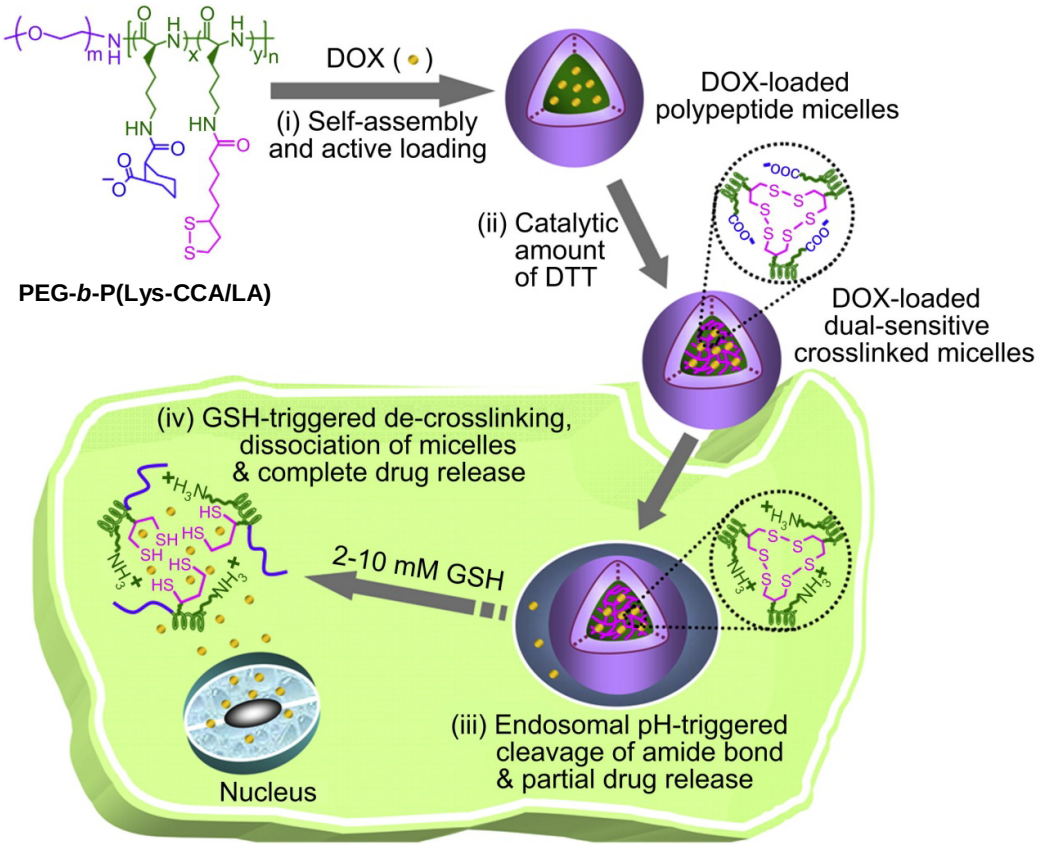


Figure 18. Schematic representation of reduction and pH dual-responsive cross-linked PEG-b-P(Lys-CCA/LA) micelles for triggered intracellular release of doxorubicin (DOX). (i) Self-assembly of PEG-P(Lys-CCA/LA) copolymer and loading of DOX; (ii) cross-linking of micelles with catalytic amount of 1,4-dithiothreitol (DTT) to yield reduction and pH dual-sensitive cross-linked micelles, (iii) endosomal pH-triggered cleavage of amide bond of CCA and partial drug release; and (iv) GSH-triggered de-cross-linking, micelle dissociation and complete drug release. Picture reprinted from the literature.[147]

Enzymatically degradable DDS rely on esterases and proteases that cleave esters or small peptide sequences which are incorporated in the DDS. A straightforward approach for designing such a DDS is the use of polyester segments in block copolymers. Mao and Gan prepared copolymer micelles using poly(glycerol-*b*-caprolactone) (lPG-*b*-PCL) and encapsulated pyrene.[154] Subsequent treatment with a lipase led to a decrease of fluorescence intensity associated with the release and aggregation-induced quenching of pyrene. This result is astonishing insofar that the interior polyester block is well shielded by the poly(glycerol) shell. Most likely the enzyme can still degrade the polyester due to the dynamics of aggregation and disaggregation. Fischer et al. attached spermine, spermidine, and pentaethylenhexamine via a carbamate linker to dPG amine.[62] These unimolecular core-shell nanocarriers were enzyme degradable and able to complex siRNA for gene delivery. Their cargo could be released with the help of two different lipases. Overall, the nanocarriers showed a lower cytotoxicity and high siRNA transfection efficiency in vitro compared to the commercial standard HiPerFect®. dPG with a PEG shell that was co-functionalized with aromatic units to increase the hydrophobicity of the core could release pyrene upon treatment with CAL B.[155] The lipase cleaves the ester groups between dPG and the aromatic unit and thereby lowers the hydrophobicity.

The manifold responsive DDS that have been developed and thoroughly studied reveal the high interest in this field. All responsive DDS were superior to their non-responsive counterparts under the given experimental setups. This proves that the introduction of responsiveness is a key step in satisfying the need for new smart DDS.

1.3 Dermal and Transdermal Drug Delivery

The skin is the biggest human organ and has many complex functions. Its major role is to provide a protective barrier. It minimizes water loss and prevents the invasion of threats from the environment, e.g., microbes, toxic agents, irradiation, and particulate matter, into the organism. For this purpose, the skin has several defensive mechanisms based on physical, immunological, metabolic, and UV-protective barriers,[156] which prevent most nanoparticles, like viruses, bacteria, dust, allergens, or materials from penetrating the skin unless it is disrupted due to injury or disease. On the other hand, once the defense mechanisms are understood, the skin could be used for the dermal or transdermal delivery of drugs. Overcoming the skins barriers in a safe and efficient way still remains a challenge in dermal and transdermal drug delivery.[157]

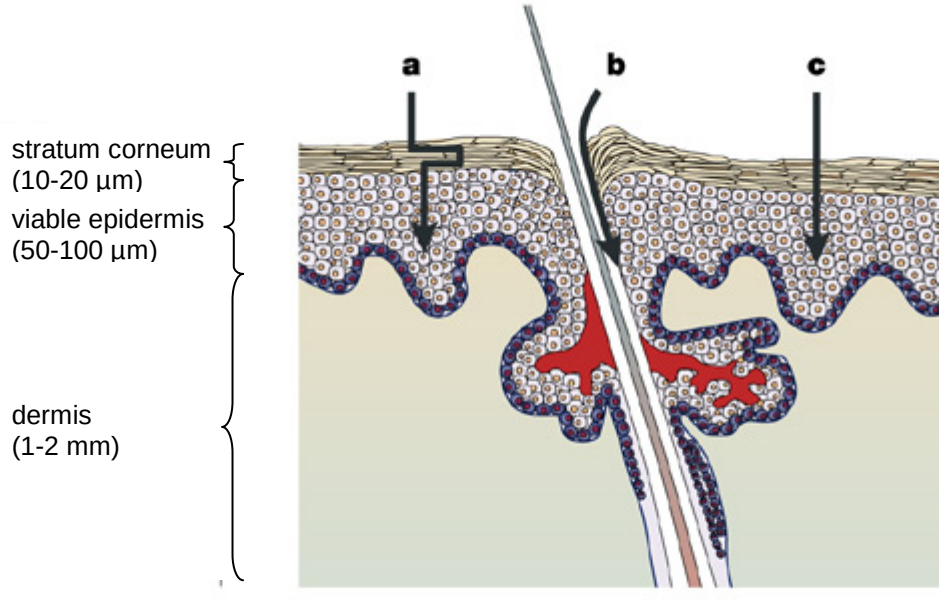


Figure 19. Schematic representation of the structure of the skin. Three pathways for crossing the stratum corneum are shown: a) the intracellular, b) the shunt, and c) the transcellular pathway. Figure adapted from the literature.[158]

As shown in Figure 19, the skin consists of two main layers: the underlying dermis is formed by a variety of cell types, nerves, blood vessels, and a lymphatic system, which are hold together by connective tissue. On top of the dermis, separated by a membrane layer, lies the epidermis that is mainly composed of stratified keratinocytes surrounded by an extracellular lipid matrix. The outermost layer of the epidermis, the stratum corneum, is the

most important one for the barrier function of the skin. Diffusion through the stratum corneum is for most substances the rate limiting step of skin permeation. Three different routes have been considered for crossing the stratum corneum – the transcellular, the intercellular, and the shunt route, involving hair follicles and sweat ducts (see Figure 19).[156, 158] It was found that polar solutes rather permeate via the transcellular route while more lipophilic solutes enter via the intercellular route through the surrounding lipids.[159] Transdermal transport of polar solutes can as well occur via the shunt pathway which allows the diffusion across the stratum corneum and the remaining epidermis (viable epidermis). Since the skin surface is much higher than the area covered by hair, transport via the stratum corneum remains the major route. All drugs that are currently administered across the skin exhibit the following properties: low molecular mass (below 500 Da), high lipophilicity, and small required dose. The delivery of bigger hydrophilic drugs into the skin remains a big challenge.[158]

Besides several nano- and microparticles that have been used for dermal and transdermal drug delivery,[156] dendrimer-mediated drug delivery gained increasing interest in recent years.[160] Although PAMAM dendrimers did not penetrate the stratum corneum, they do enhance the drug delivery into the skin to a certain extent. Three possible mechanisms have been proposed. The first possibility is that dendrimers might function as drug release modifier and speed up the drug dissolution. Here, the dendrimers help to keep the drug in a well solubilized state that is of high thermodynamic activity, boosting the drug permeation. Secondly, they might help to target the hair follicles. Despite the fact that only a low area is covered with hair, the high vascularization and deep invagination of the hair follicles makes this route interesting.[161] Finally, dendrimers can perturb the lipid bilayers impairing the stratum corneum.

Küchler et al. successfully used CMS nanocarriers developed in the Haag group to enhance the skin penetration of the hydrophilic dye rhodamin B (see Figure 20) and the hydrophobic dye Nile red.[99-100] The penetration of both guests could be significantly enhanced compared to a cream application. The CMS nanocarriers were even superior to solid lipid nanoparticles (SLN), which are considered the gold standard for drug delivery to the skin. Furthermore, CMS nanocarriers were studied concerning their tolerability by skin and it was found that they are non-toxic, non-irritant, and do not interfere with cell migration.[162] These findings prove CMS nanocarriers to be excellent candidates for DDS targeting the skin.

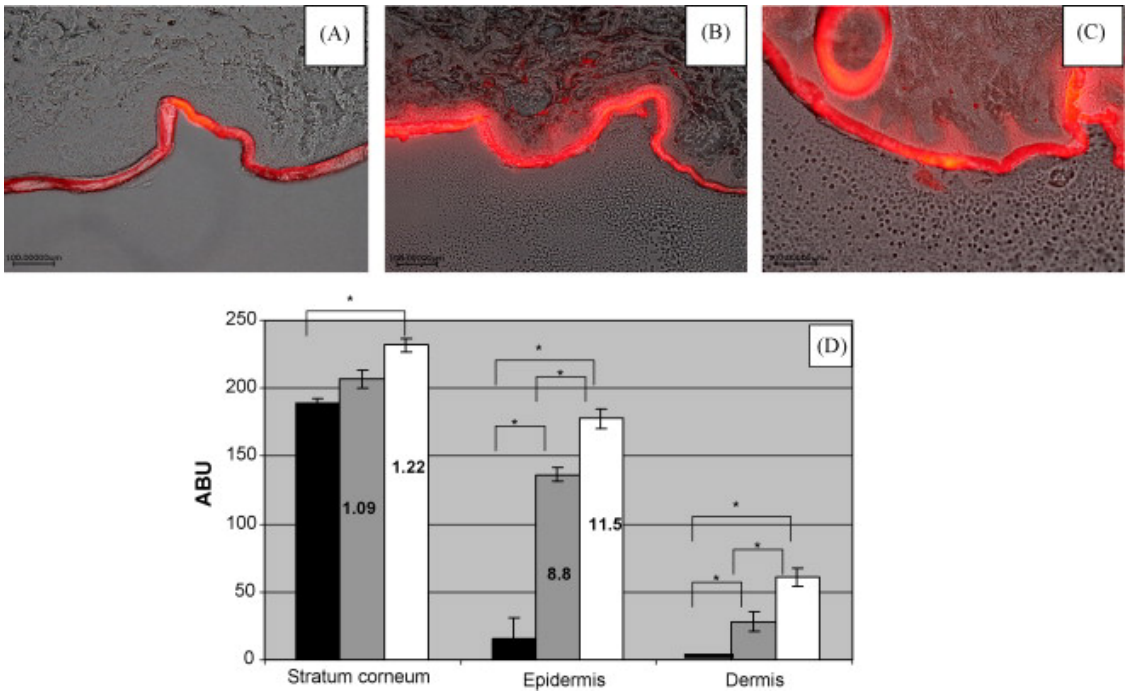
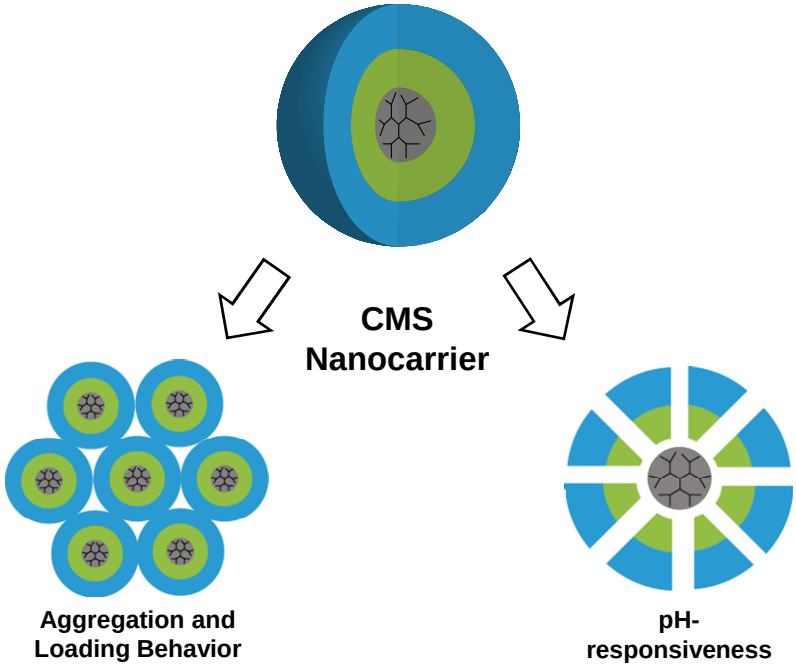


Figure 20. Rhodamin B penetration into pig skin: staining of pig skin following the application of 0.004% rhodamin B loaded cream (A), SLN (B), and CMS nanotransporters (C) for 6 h. The representative pictures taken from the identical donor animal are obtained by superposing normal light and fluorescence images of the same area. (D) The arbitrary pixel brightness values (ABU) obtained by fluorescence picture analysis (cream, black columns; SLN, grey columns; CMS nanotransporters, white columns, n = 3). The inserted numbers give the respective enhancement of penetration over cream, *differences ($p \leq 0.05$). Figure reprinted from the literature.[99]

2 Scientific Goals

Polymeric drug delivery systems (DDS) can significantly enhance the performance of various active agents for the treatment of a variety of diseases. Particularly promising DDS are CMS nanocarriers based on dPG with an inner alkyl and an outer mPEG shell. Due to their high versatility they have already been used for different biomedical applications. Particularly, their use as skin penetration enhancers shows high potential.

This work will further investigate the behavior of CMS nanocarriers upon the interaction with guest molecules. Special focus will be given to the aggregation behavior of the CMS nanocarriers when loaded with guest molecules, because the size of a DDS plays an important role in different biological events like cellular uptake. There is evidence that a branched outer shell might be suitable to prevent aggregation of CMS nanocarriers and these findings will be applied to the developed CMS systems. Until now, CMS nanocarriers were not applicable for controlled release of loaded drugs. Therefore it is desirable to develop CMS nanocarriers that respond to external stimuli.



In order to gain a better understanding of the behavior of CMS nanocarriers, their behavior upon interaction with solvatochromic dyes like Nile red and Coumarin 153 will be studied. The solvatochromic dyes allow the localization of the dye as its absorption is shifted in correlation to the polarity of the environment of the dye. Both dyes are rather hydrophobic

which might have an influence on the aggregation of the nanocarriers. The evaluation of these properties will be achieved by using UV/Vis spectroscopy, fluorescence spectroscopy, and dynamic light scattering (DLS). To test the influence of a branched outer shell, CMS nanocarriers with defined branched structures on the outside shall be synthesized. Here, PG dendrons will be used as PG has comparable properties to the previously used mPEG. Again UV/Vis spectroscopy and DLS will be used for the investigation of the aggregation behavior and determination of the transport capacity of the guest molecules. The pH was chosen as the stimulus that should trigger the release from CMS nanocarriers as this stimulus is naturally occurring during cellular uptake. More precisely, an aromatic imine group shall be introduced into the CMS nanocarrier structure. This linker is cleaved in the desired pH-range that is present after cellular uptake. Furthermore, the release of active agents from this novel nanocarrier type shall be studied.

Finally, additional information about the skin penetration enhancement by CMS nanocarriers shall be gained using electron paramagnetic resonance spectroscopy and the comparison with a different DDS.

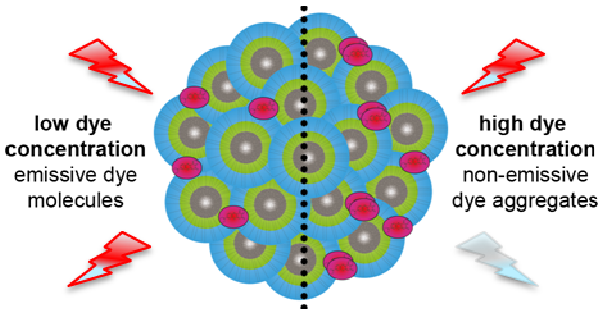
3 Publications and Manuscripts

3.1 Aggregation Phenomena of Host and Guest upon the Loading of Dendritic Core-Multishell Nanoparticles with Solvatochromic Dyes

This chapter was published in:

E. Fleige, B. Ziem, M. Grabolle, R. Haag, and U. Resch-Genger, *Macromolecules* **2012**, *45*, 9452-9459.

http://dx.doi.org/10.1021/ma301977r



Author Contribution:

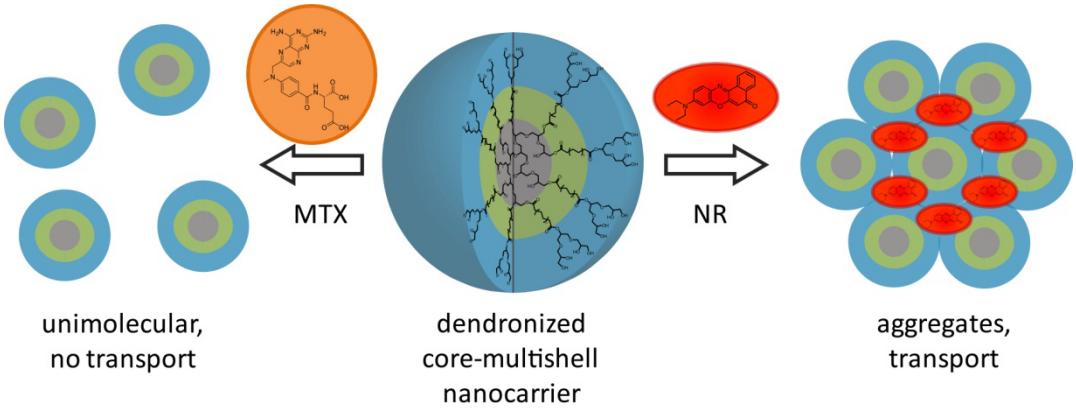
- Synthesis of the core-multishell nanocarriers
- Loading of the core-multishell nanocarriers with Nile red
- Design of the UV/Vis and fluorescence experiments
- Dynamic light scattering measurements
- Preparation of the manuscript

3.2 Dendronized Core-Multishell Nanocarriers for Solubilization of Guest Molecules

This chapter was published in:

E. Fleige, R. Tyagi, and R. Haag, *Nanocarriers* **2013**, *1*, 1-9.

http://dx.doi.org/10.2478/nanca-2013-0001



Author Contribution:

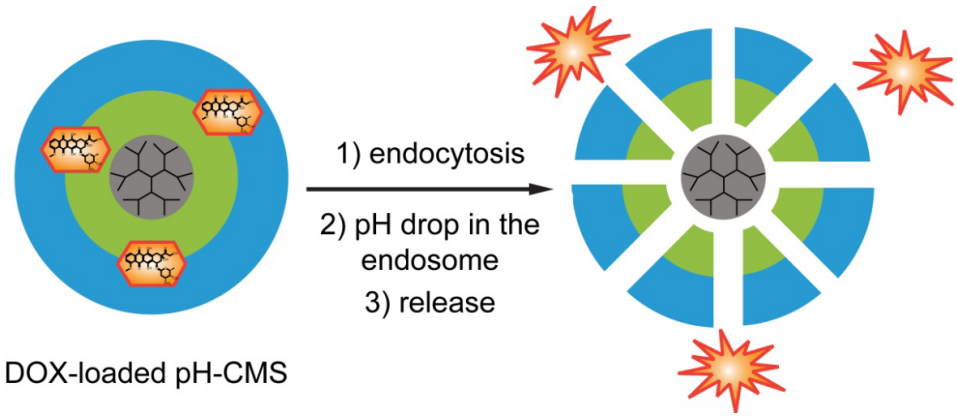
- Synthesis of the dendronized core-multishell nanocarriers with PG[G1] shell
- Loading of all core-multishell nanocarriers
- UV/Vis experiments for the determination of the transport capacities
- Dynamic light scattering experiments
- Preparation of the manuscript

3.3 pH-Responsive Dendritic Core-Multishell Nanocarriers

This chapter was published in:

E. Fleige, K. Achazi, K. Schaletzki, T. Triemer, and R. Haag, *J. Controlled Release* **2014**, *185*, 99-108.

http://dx.doi.org/10.1016/j.jconrel.2014.04.019



Author Contribution:

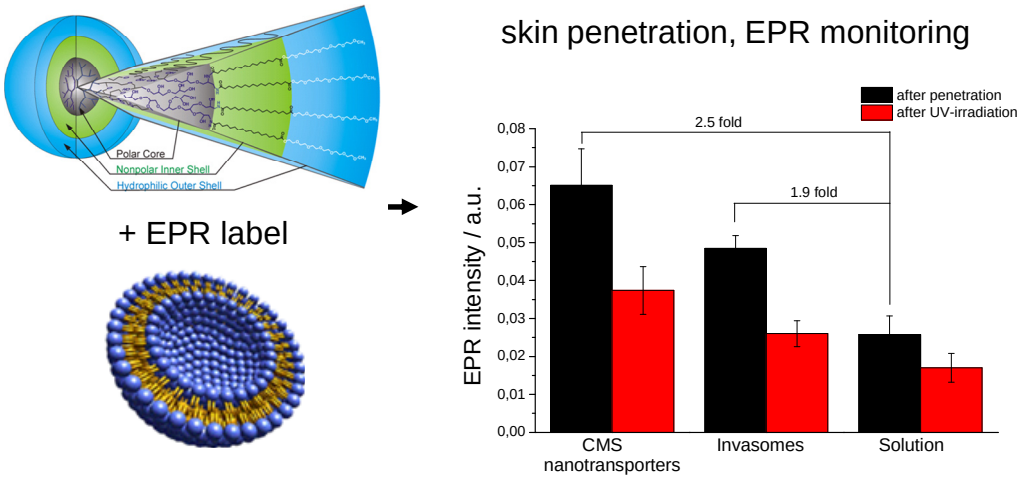
- Synthesis of the pH-responsive core-multishell nanocarriers
- Supervision of Karolina Schaletzki and Therese Triemer working on the establishment of alternative synthetic pathways
- Loading of the pH-responsive core-multishell nanocarriers
- UV/Vis experiments for the determination of the transport capacities
- Dynamic light scattering experiments
- Design of the in vitro experiments with the help of Katharina Achazi
- Preparation of the manuscript

3.4 Skin Penetration Enhancement of Core-Multishell Nanotransporters and Invasomes Measured by Electron Paramagnetic Resonance Spectroscopy

This chapter was published in:

S. F. Haag, E. Fleige, M. Chen, A. Fahr, C. Teutloff, R. Bittl, J. Lademann, M. Schäfer-Korting, R. Haag, and M. C. Meinke, *Int. J. Pharm.* **2011,** *416*, 223-228.

http://dx.doi.org/10.1016/j.ijpharm.2011.06.044



Author Contribution:

- Synthesis of the core-multishell nanocarriers
- Loading of the core-multishell nanocarriers with the EPR label
- Discussion and evaluation of the results

4 Summary and Conclusion

This work investigated the applicability of novel CMS nanocarriers as drug delivery systems. Special focus was given to the aggregation behavior of the nanocarriers upon their interaction with different guest molecules. For this purpose, CMS nanocarriers with a branched outer shell were synthesized and the influence of the branched outer shell on the aggregation and transport behavior of the nanocarriers was investigated. Furthermore, pH-responsive CMS were synthesized and their intracellular drug release profile was evaluated in comparison to a stable CMS nanocarrier.

In order to investigate the loading of CMS nanocarriers with the solvatochromic dyes Nile red and Coumarin 153 spectroscopic studies were performed. It was found that the spectroscopic properties and the size of the dye-loaded CMS nanocarriers are controlled by the hydrophilicity and aggregation behavior of the incorporated fluorophores. The sensitivity of the dye's absorption and emission depends on the polarity of its immediate environment and on hydrophobic effects controlling the nanocarrier aggregation. Furthermore, it was shown by absorption and fluorescence spectroscopy that the internalization of these hydrophobic and solvatochromic dyes occurs within the outer layer of the CMS nanocarriers. The uptake of Nile red by CMS nanocarriers enhances the aggregation of the dye as indicated by hypsochromically shifted absorption bands and a concentration-dependent loss in fluorescence. This represents one of the very few examples of the formation of H-type aggregates of Nile red. For Coumarin 153 loaded CMS nanocarriers, a red shift in absorption, a blue shift in emission, and a diminution of the fluorescence quantum yield was observed upon increasing the dye loading concentration. This seems to derive from a new aggregate species of the dye with properties that have not been observed before. The loading of the CMS particles with both dyes led to an enhanced CMS nanocarrier aggregate formation.

In addition, a new type of dendronized CMS nanocarrier with PG dendrons of different sizes as the outer shell was synthesized and compared to CMS nanocarriers with a linear mPEG outer shell. The CMS nanocarriers with PG dendrons of the 1st generation showed an increased transport capacity of Nile red. The 2nd generation, however, only achieved transport capacities comparable to the values obtained for mPEG-terminated CMS nanocarriers. In case of the drug methotrexate, only mPEG-terminated CMS nanocarriers were able to solubilize the guest. Nile red-loaded CMS nanocarriers exclusively formed aggregates, while the methotrexate-loaded carriers showed sizes corresponding to single CMS nanocarriers. It was shown that the formation of CMS nanocarrier aggregates cannot be

prevented by replacing the mPEG outer shell with branched PG dendrons. Furthermore, it was concluded that the aggregation behavior is strongly influenced by the guest molecule.

Moreover, a pathway for the synthesis of pH-responsive CMS nanocarriers was established. These responsive nanocarriers release doxorubicin more easily after cellular uptake and therefore show higher toxicity in comparison to the corresponding stable carriers. The transport of doxorubicin was achieved in unimolecular CMS nanocarriers, which again showed that the guest has a strong influence on the aggregation behavior.

Finally, it was confirmed that CMS nanocarriers are efficient drug delivery systems for the topical application of hydrophilic substances to the skin. In direct comparison with lipid-based invasomes, the CMS nanocarriers show a higher penetration enhancement especially in the upper layers of the stratum corneum. Furthermore, it was shown that the used EPR-label was still active after penetration and reacted with free radicals in the skin generated by UV-irradiation.

5 Outlook

The versatility of CMS nanocarriers makes them an extremely interesting candidate for the design of novel drug delivery systems. With the possibility of introducing responsiveness towards external stimuli they have the chance to become outstanding smart delivery devices for the controlled application of various active agents. Furthermore, it should be possible to attach targeting moieties to the surface of CMS nanocarriers. In that case they would not only take advantage of the EPR effect for passive targeting but in addition would benefit from active targeting as well which would increase their specificity. However, in order to increase their functionality, a more and more tedious synthesis is required and makes the CMS nanocarriers more expensive. Taking this into account, it seems unreasonable to expect the production of large quantities of responsive CMS nanocarriers with targeting moieties and their market approval as DDS in the near future. Nevertheless, already non-responsive, passively targeting CMS nanocarriers represent interesting DDS. Especially their use as skin penetration enhancers is highly promising as they outrun the so-called solid lipid nanoparticles, which are considered the gold standard at the moment. In addition, the investigation of the actual guest properties that either cause or prevent the aggregation of CMS nanocarriers should provide valuable information leading to a structure-property relationship for the rational design of DDS. This would be of high importance since these factors can have a direct influence on the uptake of the delivery system, its release behavior, and the activity of the active agent.

6 Zusammenfassung

Ziel dieser Arbeit war die Entwicklung und Evaluierung neuartiger Kern-Multischale-Architekturen als Nanotransportsysteme für Wirkstoffe. Zum einen sollte die Wechselwirkung der Trägermoleküle mit verschiedenen Gastmolekülen untersucht werden und zum anderen sollte ein spaltbares System konzipiert werden, das eine gezielte Freisetzung der Gäste ermöglicht. Zur Evaluierung der Wechselwirkungen zwischen Kern-Multischalen-Nanocarriern und verschiedenen Gastmolekülen wurden Trägersysteme sowohl mit linearer als auch verzweigter Außenschale hergestellt und der Einfluss der Schalenarchitektur auf das Aggregations- und Transportverhalten der Kern-Multischale-Nanocarrier untersucht. Zusätzlich wurden die Kern-Multischale-Nanocarrier mit pH-empfindlichen Bindungen zwischen Kern und Schale ausgestattet und ihr intrazelluläres Wirkstoff-Freisetzungsprofil im Vergleich zu stabilen Kern-Multischale-Nanocarriern untersucht.

Mit Hilfe spektroskopischer Methoden wurde die Beladung der Kern-Multischale-Nanocarrier mit den solvatochromen Farbstoffen Nilrot und Coumarin 153 untersucht. Dabei wurde festgestellt, dass die spektroskopischen Eigenschaften und das Aggregationsverhalten der farbstoffbeladenen Transportsysteme durch die Polarität und das Aggregationsverhalten der verwendeten Farbstoffe bestimmt werden. Die Absorption und Emission der Farbstoffe wurden dabei stark von der Umgebung des Farbstoffes und hydrophoben Effekten beeinflusst. Diese haben auch einen Einfluss auf das Aggregationsverhalten der Kern-Multischale-Nanocarrier. Die hydrophoben und solvatochromen Farbstoffe wurden dabei insbesondere durch die äußere Schale der Kern-Multischale-Nanocarrier aufgenommen, was mit Hilfe von UV/Vis- und Fluoreszenzspektroskopie bewiesen werden konnte. Bei der Aufnahme von Nilrot in die Nanotransporter konnte eine Selbstaggregation des Farbstoffes beobachtet werden, die sich in einer hypsochromen Verschiebung der Absorptionsbande und einer konzentrationsabhängigen Fluoreszenzquenchung zeigt. Dies stellt eines der wenigen Beispiele dar, bei dem sogenannte H-Aggregate für den Farbstoff Nilrot beobachtet werden konnten. Die mit Coumarin 153 beladenen Kern-Multischale-Nanocarrier zeigten eine Rotverschiebung in der Absorption, jedoch eine Blauverschiebung in der Emission und ein Verminderung der Fluoreszenzquantenausbeute, die in Abhängigkeit von der Farbstoffkonzentration stehen. Diese Eigenschaften scheinen von einem neuartigen Coumarin 153-Aggregat herzurühren, das in dieser Form noch nicht beobachtet wurde. Die Beladung der Kern-Multischale-Nanocarrier führte bei beiden Farbstoffen zu einer verstärkten Aggregatbildung der Nanotransporter.

Desweiteren wurde eine neue Art von Kern-Multischale-Nanocarriern hergestellt, die statt linearem mPEG unterschiedlich große PG-Dendronen als äußere Schale aufweisen. Durch die Verwendung von PG-Dendronen der ersten Generation konnte die Transportkapazität mehr als verdoppelt werden. Die Nanotransporter mit Dendronen der Generation zwei wiesen hingegen nur eine ähnlich hohe Transportkapazität wie die Kern-Multischale-Nanocarrier mit mPEG-Schale auf. Der Wirkstoff Methotrexat konnte allerdings nur mit den mPEG-funktionalisierten Nanocarrier transportiert werden. Während die mit Nilrot beladenen Kern-Multischale-Nanocarrier ausschließlich Aggregate gebildet haben, wurde durch Methotrexat die Tendenz zur Aggregatbildung verringert und es konnten nur einzelne Kern-Multischale-Nanocarrier nachgewiesen werden. Dies zeigt, dass die Aggregatbildung nicht durch die Verwendung verzweigter Bausteine in der äußeren Schale verhindert werden kann. Vielmehr lässt das den Schluss zu, dass die Aggregatbildung hauptsächlich vom Gastmolekül beeinflusst wird.

Ein weiterer Teil der Arbeit beschäftigte sich mit der Herstellung von pH-empfindlichen Kern-Multischale-Nanotransportern. Dies wurde durch Einführung einer Imingruppe zwischen Kern und Doppelschalenbaustein erreicht, die so erhaltenen pH-empfindlichen Nanocarrier konnten Doxorubicin erfolgreich verkapseln und nach zellulärer Aufnahme wieder freisetzen. Durch die aktiv ausgelöste Freisetzung zeigten die Nanocarrier eine höhere Zelltoxizität als das stabile Vergleichssystem. Der Transport von Doxorubicin fand ausschließlich durch unimolekulare Kern-Multischale-Nanocarrier statt. Dies zeigt wiederholt, dass die Aggregation der Nanocarrier stark von der Art des zu transportierenden Moleküls abhängt.

Zu guter Letzt konnte auch gezeigt werden, dass die entwickelten Kern-Multischale-Nanocarrier sehr effektiv als Wirkstofftransporter für die topische Anwendung hydrophiler Substanzen auf der Haut verwendet werden können. Im direkten Vergleich mit lipidbasierten Invasomen konnte mit den Kern-Multischale-Nanotransportern eine deutlich höhere Hautpenetration, insbesondere in die oberen Schichten des Stratum corneum, erreicht werden. Außerdem konnte gezeigt werden, dass das eingebrachte EPR-Label nach Penetration noch aktiv war und mit freien Radikalen in der Haut reagieren konnte, die durch UV-Strahlen generiert wurden.

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