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Core-shell and core-amphiphilic branched shell nanocarriers based on biodegradable hyperbranched polymers, as potential drug delivery systems

INAUGURAL-DISSERTATION

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The following Ph.D. thesis was supervised by Dr. Paul Servin and carried out inside Nanopartica GmbH and in tight collaboration with the research group of Prof. Dr. Rainer Haag from July 2012 until January 2016 at the Department of Biology, Chemistry, and Pharmacy of the Freie Universität Berlin. Nanopartica GmbH and IGSTC have supported the first three years of studies. The final semester has been funded by SFB 1112.

1st Reviewer: Prof. Dr. Rainer Haag

2nd Reviewer: Jr. Prof. Marcelo Calderón

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...to our little angel,

Edoardo.

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Abbreviations

AA	adipic acid
ABS	amphiphilic branched shell
AFM	atomic force microscopy
ASA	alkenyl succinic anhydride
CAL B	Candica Antartica lipase B
CMC	critical micelle concentration
CMS	core-multishell
D	see PDI
DB	degree of branching
DDCA	dodecanedicarboxylic acid
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DDS	drug delivery system
DLS	dynamic light scattering
DMAP	dimethyl amino pyridine
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOX	doxorubicin
DXM	dexamethasone
dPG	dendritic polyglycerol
EDC-HCl	1-Ethyl-3-(3- dimethylaminopropyl)carbodiimide hydrochloride
EPR	enhanced permeation and retention
FDA	US Food and Drugs Administration
FNS	finasteride
GPC	gel permeation chromatography
GSH	glutathione
HaCaT	cultured human keratinocytes (cells)
HBPE	hyperbranched polyester
HIV	human immunodeficiency virus
HPLC	high-pressure liquid chromatography
IR	infrared spectroscopy
LC	loading capacity
mPEG	mono methyl poly(ethylene glycol)

Mn	number average molecular weight
Mw	weight average molecular weight
MTT	3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio
MWCO	molecular weight cut-off
NMR	nuclear magnetic resonance
PAMAM	poly(amido amine)
PCL	poly(e-caprolactone)
PDI	polydispersity index
PEG	poly(ethylene glycol)
PEGylation	conjugation of PEG to a functionalized matter
PEI	poly(ethylene imine)
PPI	poly(propylene imine)
ppm	parts per million
PTFE	poly(tetrafluoro ethylene)
PTSA	para-toluenesulfonic acid
RNA	ribonucleic acid
ROMB	ring opening multibranching polymerization
r.t.	room temperature
SA	succinic acid
siRNA	small interfering RNA
TEM	transmission electron microscopy
THF	tetrahydrofuran
TMP	1,1,1-tris (hydroxymethyl) propane
wt-%	weight percent

1. Introduction

1.1 Polymeric Drug Delivery Systems

The vast majority of commercialized medicines contain a polymer within their formulation. Polymers thus play an important role in the pharmaceutical field. For example, they act as wicking and disintegration components of tablets, lubricants, enteric coatings, wetting agents, solid dispersion phases, penetration enhancers, and modifiers of viscosity.[1] Despite these uses, in the last decade, polymers have gained enormous importance in the field of medicine as potential drug delivery systems (DDS). In more general terms, a polymeric DDS is polymer engineered matter which is able to transport, and release, biologically active compounds or drugs inside the body. One of the great advantages of these systems is that a DDS can be targeted for a specific disease, in order to transport and release the drug at a predetermined location e.g. a tumour, inflamed tissue or a specific organ.[2]

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. They are distributed all over the human body and can easily diffuse into healthy tissue with the direct consequence of often displaying remarkable side effects, thus allowing only a small amount of the drug to reach the targeted site. High doses or repetitive injections are required to reach the therapeutic dose at the target site with the risks of alternating times when the concentration of the drug in the body is above the therapeutic window (increased side-effects) and below the therapeutic window (inactivity of the drug) (Figure 1).

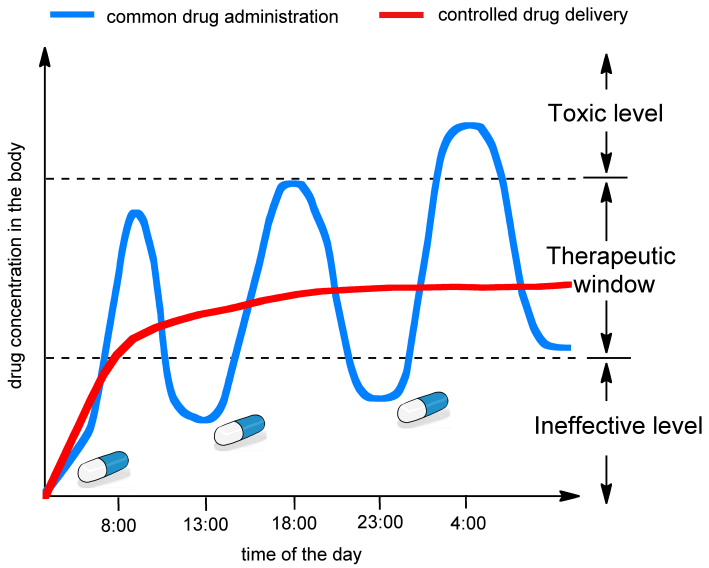


Figure 1. Comparison of drug concentration in the body of a drug administered several times (blue curve) with a controlled drug delivery system showing idealized pharmacokinetics (red curve).

An ideal DDS overcomes this problem by allowing a controlled and constant release of the drug inside the body which, when targeted to the desired location, maximizes the activity of the drug, therefore decreasing the dosages.
Furthermore the DDS can modify and boost the intrinsic characteristics of the drug by increasing the solubility of lipophilic substances, their biocompatibility and stability by shielding its toxicity or by protecting the active molecule from the external environment until it reaches the targeted site of action.
The major reasons that call for the use of a drug delivery system and therefore constantly fuel research to develop new and more efficient systems are given as follows:[4]

- Dissolution of a drug is too slow
- Drug and/or formulation is physically removed from the site of action too quickly
- Metabolism or excretion of the drug is too fast
- Avoiding rapid metabolism or excretion from the body
- Accelerating or avoiding transporting the drug across certain cell membranes
- Avoiding side effects by minimizing exposure of other tissue
- Concentrating drug at the site of action
- Drug is required intermittently
- Administration is complex, invasive, and/or costly
- Patient compliance (i.e. motivation to remember to take dosage) is poor and consequences of missed dosages are serious

Therefore, all DDS should fulfill one or more of the following points in order to enhance the availability of the active agent.[5,6]

- 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 therefore reducing its systemic toxicity
- Release the drug in response to an external stimulus present at the site of action or locally applied from outside of the body

One major challenge connected with the use of these systems is the elimination of the carriers from the body after the drug has been transported and released. On the one hand the DDS must be stable until the targeted site is reached; on the other hand, the DDS should be, after liberation of the guest, excreted from the body or degraded in order to avoid an undesired accumulation in organs. DDSs based on biodegradable polymers that, in presence of enzymes or acidic pH, undergo a backbone breakdown, allow for the release of the encapsulated guest molecules and their easy clearance from

the body. Due to the suitable characteristics of these systems made of biodegradable DDSs, in the last decade, they have become the most prominent studied systems.

The size of the non-biodegradable polymeric DDS is of fundamental importance in order to allow for its elimination from the body. It is well known that particles of sizes below 200 nm can avoid recognition from the reticuloendothelial system and can therefore allow a longer circulation time in the blood systems and particles with sizes below 6 nm, can be excreted from the kidneys, and therefore eliminated from the body.[7,8,9]

The advantages brought about by nanometre-scale DDSs in allowing for a longer blood half-life time and the possibility of being easily excreted from the body placed the research within the field of so-called nano-DDSs even referred to as nanocarriers.
Different types of polymers are used nowadays as nanocarriers. Figure 2 displays the most important classes: polymeric micelles, formed after aggregation of linear or grafted amphiphilic polymers, functionalized dendritic polymers that include dendrimers and hyperbranched polymers, and nanogels, which are macrostructures composed by a cross-linked polymeric network.

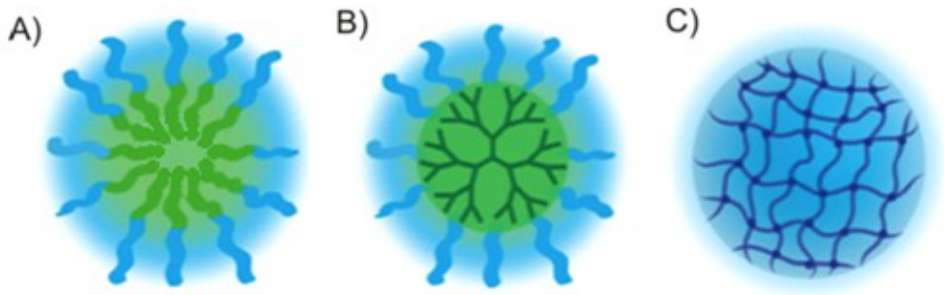


Figure 2. Schematic representation of different DDSs; A) polymeric micelle, B) functionalized dendritic polymer and C) nanogel. Figure adapted from literature [3].

This work focuses on the dendritic polymer and in particular on hyperbranched polymers and their uses as drug nanocarriers, which is the subject of this thesis; polymeric micelles and nanogels will not be discussed in detail, in spite of their considerable interest in a wider context.

1.2 Dendritic Drug Delivery Systems

Dendritic macromolecules, named from the Greek word for tree (*dendron*), are characterized by a highly branched tree-like globular structure. Dendrimers were investigated for the first time by Flory in 1941,[10,11,12] however the first report regarding those macromolecules appeared in 1978 with the work of Vögtle and co-workers.[13] Since then, several classes of dendritic structures have been developed and can be divided into 4 major classes: (a) dendrimers, (b) dendrons, (c) dendronized polymers, and (d-e) hyperbranched polymers (Figure 3).

Dendrimers have perfectly branched architectures characterized by a polydispersity index (PDI) of one (flowing chapter). This means that the molecules are monodisperse. Dendrons, which can be considered as one branch of a dendrimer, also exhibit perfect structures but are characterized by a functionalized focal point from where the branched structure grows perfectly. In contrast, dendronized polymers and hyperbranched polymers are polydisperse architectures. Dendronized polymers, also referred to as dendrigrafts, are commonly composed of a functionalized linear polymer as a backbone with dendrons attached to it. Hyperbranched polymers possess a similar architecture to dendrimers, but are characterized by higher PDIs (typically around 1.5-3). These macromolecules in fact have an imperfect branched structure.

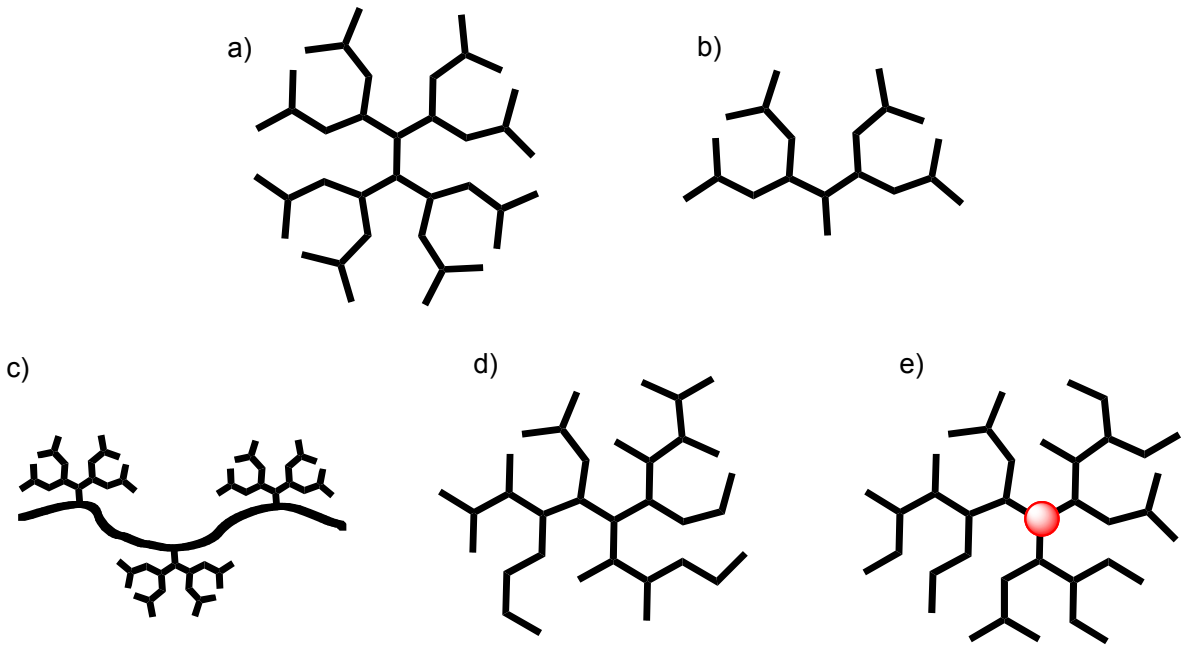


Figure 3. Schematic representation of different dendritic polymers: a) dendrimer, b) dendron, c) dendronized polymer d) hyperbranched polymer and e) hyperbranched polymer synthetized using a central initiator molecule.

Furthermore, they can be divided in two classes depending on the synthesis procedure (d and e); it is possible to produce a hyperbranched polymer via random polymerization of a polyfunctional monomer (d), or by using an initiator molecule from where the polymeric backbone is grown (e). In some cases, the importance of a central molecule for obtaining lower polydispersity and therefore to obtain less imperfect polymeric structures could be shown.[14,15]

Among all polymers used for the development of DDS, dendrimers and hyperbranched polymers have displayed properties that are highly advantageous for use as efficient nanocarriers.[16,17,18,19]
The benefits of dendritic polymers compared to similar linear polymers are their high solubility, a low intrinsic viscosity and, most importantly, the high number of functional groups which allow various modifications.[20,21,22,23] The strategic functionalization of the external surface of these globular structures enable the modification and tailoring of the solubility, conjugation, targeting, recognition and the formation of internal cavities for encapsulation of guests. Moreover, when employed as DDSs, dendritic nanocarriers display higher stability upon dilution and/or external stimuli, compared to similar micellar structures created after the aggregation of amphiphilic linear polymers.

In the field of dendritic polymers used as drug delivery systems, two main strategies have been adopted in order to efficiently transport the active substances to the targeted site. In the first strategy, which will be further described in this work, the polymer acts as a carrier for the active substance (guest) and the entrapment is based on non-specific or specific interactions (Figure 4). The release usually occurs by diffusion of the active substance or by degradation of the carrier structure via different mechanisms. The second strategy consists of covalently binding the active substance to the polymer, to give polymer-"guest" conjugates. Release then occurs via bond cleavage.

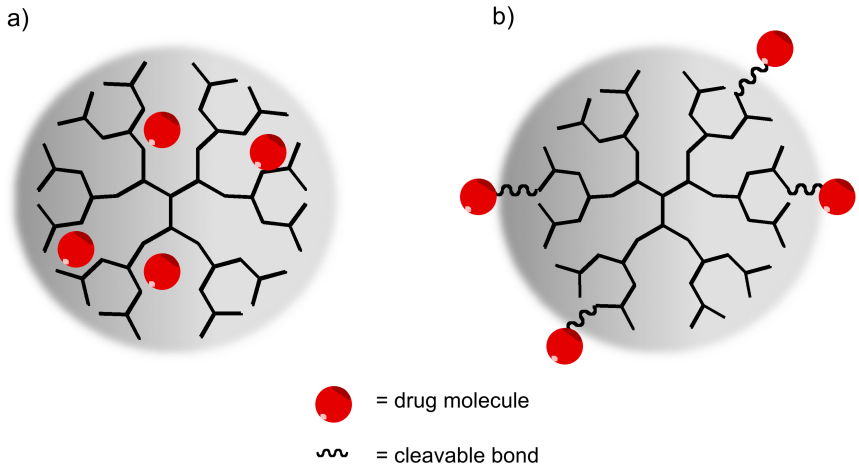


Figure 4. Different approaches for the usage of dendritic polymers as drug delivery systems: a) non-covalent/supramolecular encapsulation of the guest molecules, b) dendritic polymer-drug conjugates.

Despite chemical attachment, as mentioned above, drugs can be encapsulated into the nanocarrier through hydrophobic interaction or hydrogen bonds with the polymeric backbone.[24] Several encapsulation methodologies are employed to incorporate the hydrophobic drug molecules and solubilize them in aqueous solution. In the *dialysis method* for example, the drug and the polymeric nanocarrier are dissolved in a suitable organic solvent (possibly with a partial water affinity) and dialysis against water is used to gradually replace the organic solvent with water. The process of water replacement causes the direct incorporation of the drug, or the self-association of the polymeric structures, resulting in the encapsulation of the drug molecules within the micelle cores. The non-loaded drug is removed through the dialysis bag while the encapsulated drugs or micellar aggregates are trapped inside.[25,26] In the *oil-water emulsion method*, the drug, dissolved in a selected organic solvent, is added to an aqueous solution of the polymeric nanocarrier. While stirring, the low boiling point organic solvent is allowed to completely evaporate from the mixture resulting in the encapsulation of the drug in the nanocarrier.

Despite there being several other methodologies described in literature (*i.e. solvent or co-solvent evaporation method, freeze drying method*)[26,26] the *film method* was found to be the most versatile and reproducible on a laboratory scale. In this methodology, employed in the experiments of the present work, a predetermined amount of hydrophobic drug is dissolved in an appropriate organic solvent (Figure 5). The solvent is completely evaporated, allowing the formation of a thin and constant film of drug in the flask. An aqueous nanocarrier solution is then added and the mixture is vigorously stirred to prone the encapsulation of the drug molecules. In the end of this process, the non-loaded drug is separated from the solution via filtration or centrifugation.

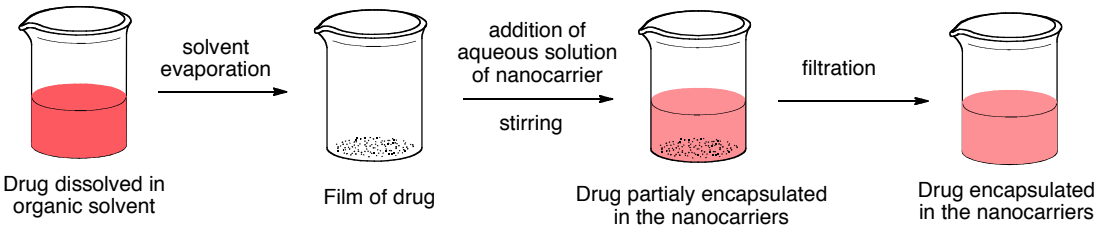


Figure 5. Schematic representation of a drug encapsulation methodology "film method".

1.2.1 Dendrimers

As previously mentioned above, dendrimers are perfect monodisperse macromolecules with a regular and highly branched three-dimensional architecture. Within the dendrimer structure itself we can distinguish three different parts: the core, the branched repeating units, and the terminal end groups sitting on the external molecular surface. The first dendrimers were synthetized during the 1980s by

Tomalia and co-workers in which the dendrimer is built up from a multifunctional core by reiterated addition of the repeating branched units.[27] These enumerated repeating layers are also called generations (*i.e.* G1, G2), which is generally the growth identification of a dendrimer. This type of growth synthesis can be made according to two approaches. The first one, named "divergent approach" (Figure 6, top), is a multi-step process, requiring protection, deprotection and purification upon addition of each generation. A second approach, known as "convergent synthesis" (Figure 6, bottom), led to an increase in the range of dendrimers with controlled structure and extreme purity. In convergent synthesis, developed in the early 1990s by Hawker and Fréchet, the dendritic arms are first synthesized and then grafted to a multifunctional core molecule.[28]

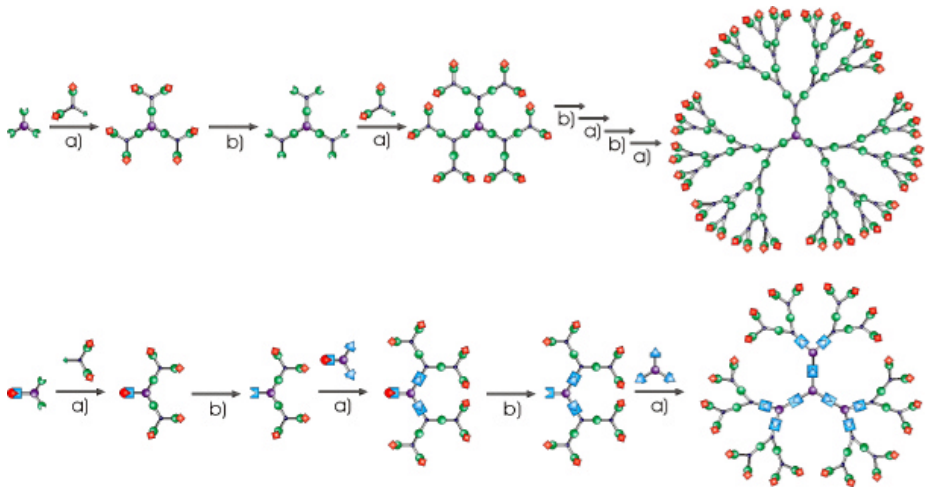


Figure 6. Divergent (top) and convergent (bottom) schematic synthesis of dendrimers. Step a) coupling. Step b) deprotection. Figure reprinted from literature [29].

The major challenge in the synthesis of dendrimers is the synthetic complexity caused by the high purity of the final product required. In the divergent approach, and in particular for higher generation dendrimers, several identical reactions have to be performed on a single molecule. Therefore, to ensure the integrity of the final product, every reaction has to be selective and proceed with at least 99.9% conversion. Due to the small differences in conformation of the perfect and the defected dendrimer, the purification step during divergent synthesis is very tedious and increases with the generations of the dendrimers. In the convergent approach, the big differences in the masses of perfect and imperfect dendrons facilitate the separation of product from side-products. Despite easier purification, the convergent synthesis of dendrimers has some limitations. Given that in the last step of the synthesis, the high molecular weight dendrons are attached to a generally small central core molecule, the consequent high sterical hindrance might affect the selectivity of the reaction.

After the great pioneering works regarding the synthesis of dendrimers, a big effort has been made to exploit these macromolecules for medical applications. New biomedical techniques and tools have

thus been developed using dendrimers and applied in gene therapy, drug delivery, photodynamic therapy, magnetic resonance imaging and solubility enhancement as nanocomposites and as homogeneous catalysts.[30,31,32]

Nowadays, the most popular and widely used dendrimers for biomedical applications are poly(propylene imine) (PPI, Astramol®) and poly(amidoamine) (PAMAM, Starburst®) (Figure 7); these dendrimers are commercially available in kilogram scale, although the unsolved problems of purification and multistep synthesis strongly influence the price and quality of the final materials.

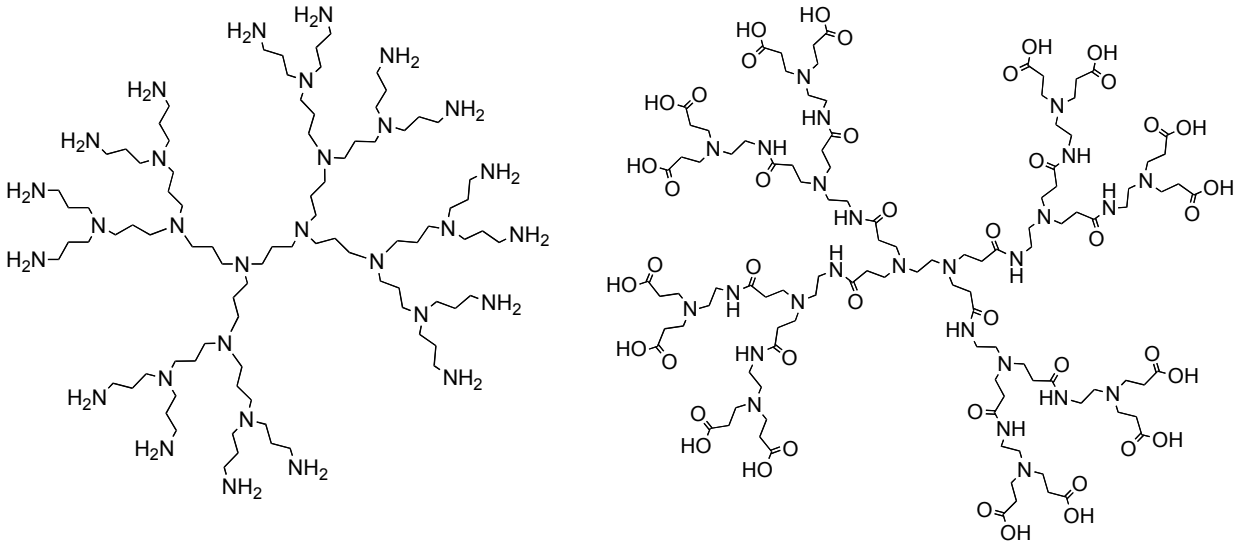


Figure 7. Structural representation of the dendrimers poly(propylene imine) (PPI, Astramol®) and poly(amidoamine) (PAMAM, Starburst®).

PAMAM has often been used as a scaffold for the covalent conjugation of drug molecules. This dendrimer enabled the solubilisation of the hydrophobic drug molecules in aqueous media and furthermore transported them into cancer cells, where they were released and accumulated in specific cell compartments.[33,34,35]

Moreover, non-covalent transport can also be induced by the ionic interactions of PAMAM with DNA strings to form stable complexes. It was even proven that the DNA/PAMAM complexes could enter cells via endocytosis thus improving transfection and enabling therefore their use in gene delivery.[36,37] Furthermore, both PPI as well as PAMAM are able to encapsulate both hydrophilic and hydrophobic drugs molecules and other active substances.[38,39]

The greater advantage of dendrimers over micelles is their high stability, independent of the dendrimer concentration. However, it has been reported that the release of the guest molecules can be

tedious; in some cases the encapsulation and release processes of the guests even resulted in an equilibrium.[40]

1.2.2 Hyperbranched polymers

Despite constant progress in the development and optimization of the synthesis of perfect dendrimers, the tedious and expensive multistep syntheses proved to be the major drawback in the commercial use of such perfectly branched macromolecules.[41]

As a valid alternative to dendrimers, hyperbranched polymers have been introduced and their development and usage has increased exponentially in the last decades. In contrast to dendrimers, these imperfectly branched polymers are generally synthesized in a one-step process.
The history of hyperbranched polymers in fact began at the end of 19th century when Berzelius reported the synthesis of a resin from tartaric acid (A_2-B_2 monomer) and glycerol (B_3 monomer).[42] In 1952, Flory *et al.* reported a theory declaring that highly branched polymers can be synthesized without gelation by polycondensation of the AB_n monomer ($n \geq 2$) in which A and B functional groups can react with each other.[43] 100 years after the first reported synthesis, Kim and Webster, upon synthesizing a soluble hyperbranched polyphenylene, named - for the first time - this type of imperfectly branched macromolecule "hyperbranched polymer".[44,45,46] Since then, hyperbranched polymers have attracted increasing attention owing to their unique properties and greater availability than their dendrimer analogues. The facile one-pot synthesis and purification, accompanied by a relatively well defined structure, are therefore the major reasons for the great interest that hyperbranched polymers produced in the last decades. In some cases, the imperfect structure of hyperbranched polymers can even be helpful for applications in drug or gene delivery and organic synthesis.[47,48,49,50,51]

There are three major methods used in the preparation of hyperbranched polymers: step-growth polycondensation of AB_n monomers ($n \geq 2$), self-condensing vinyl polymerization and ring-opening multibranching polymerization.[4,14]
The step-growth strategy was initially used, as previously mentioned above, for the synthesis of a vast range of HBPs employing different AB_n monomers. Nowadays several HBPs like Poly(phenylene), Poly(carbonate), Poly(amide), Poly(urethane) and Poly(ester) are still obtained using this procedure. The most prominent example, produced on a large scale by the Swedish company Perstorp AB (Boltorn, Figure 8), and further described by Malmstöm *et al.,* uses a polyol as a core molecule and 2,2-bis (hydroxymethyl)propionic acid (*bis*-MPA) as the AB_2 monomer.[52] The esterification is carried

out using an acid catalyst that allows to obtain a high molecular weight and degree of branching.

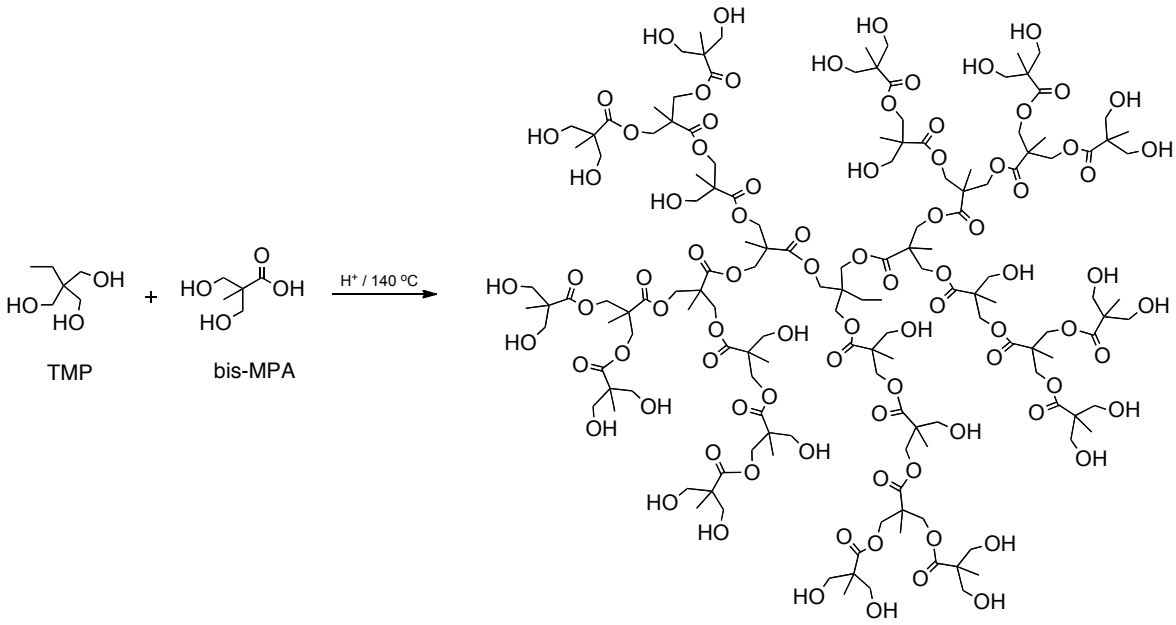


Figure 8. Synthesis of hyperbranched aliphatic polyester from bis-MPA as AB_2 monomer.

Fréchet et al. in 1995 developed the synthesis of a HBP via self-condensing vinyl polymerization of AB* type monomers.[53] The methodology involves the activation of a group associated with a double bond (A), which will react with the double bond of a second AB* monomer forming a covalent bond and a new active site (B) (Figure 9a-b). This technique has been employed to obtain Poly(styrene) and poly(meth)acrylate hyperbranched polymers.

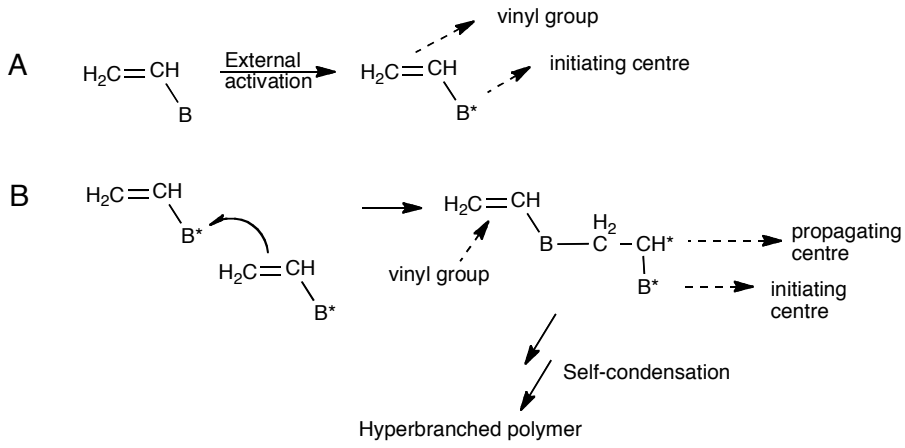


Figure 9a. Synthesis of HBP via self-condensing vinyl polymerization of AB* type monomers.

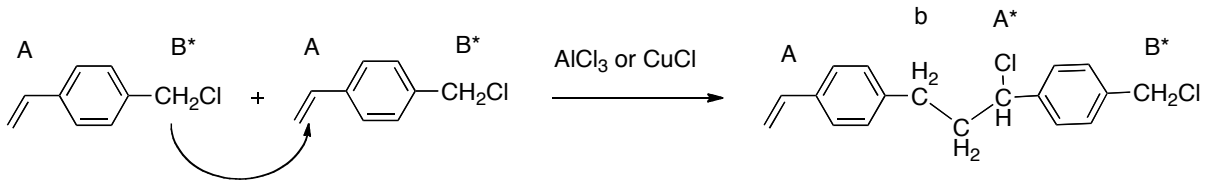


Figure 9b. Initial step of self-condensing vinyl polymerization of *p*-(chloromethyl)styrene.

The ring-opening multibranching (ROMB) polymerisation was developed by Suzuki in 1992, who synthesized hyperbranched poly(amine) from cyclic carbamate.[54] Branch points are generated through the propagation step. Cyclic epoxide, carbamate, oxetane and caprolactone have been employed as monomers for the preparation of HBPs using this methodology.

Hyperbranched polyglycerol is typically synthesized from the commercially available and highly reactive functionalized epoxide, glycidol as latent AB_2 monomer, by utilizing ROMB polymerization.[55,56] This strategy can be considered as a special polycondensation variant of AB_n monomers, where the complementary reactive groups stay latent within the ring structure.

Controlled anionic ring-opening polymerization of glycidol is generally carried out using partially deprotonated polyol (TMP) as the initiator (Figure 10). Slow addition of the monomer to allow a rapid cation-exchange equilibrium and to minimize polymerization without initiator (resulting in cyclization) favors the formation of well-defined hyperbranched polyether structure. Due to controlled polymerization conditions, the monomer exclusively reacts with the growing multifunctional hyperbranched polymer, leading to a 'living' type growth of the macromolecule.[57]

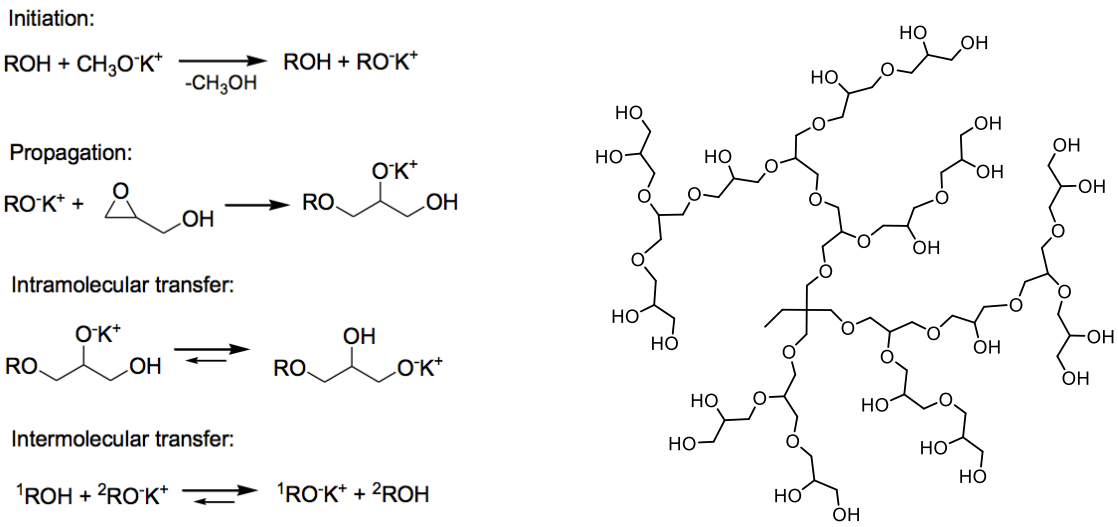


Figure 10. Mechanism of the anionic ring-opening multibranching polymerization of glycidol (left) and schematic structure representation of dPG (right).

The one-step synthesis, commonly employed for the production of hyperbranched polymers, leads to an uncontrolled statistical growth. Therefore, the structures of the branched macromolecules obtained end up being imperfect and polydisperse. As shown in Figure 11, HBPs produced by polymerization of an AB_2 monomer, consist of dendritic units (D) (all the functional groups of the AB_2 monomer have reacted), linear units (L) (one functional (B) group did not react) and terminal units (T) (both functional B groups did not react). Linear units are generally considered to be defects.

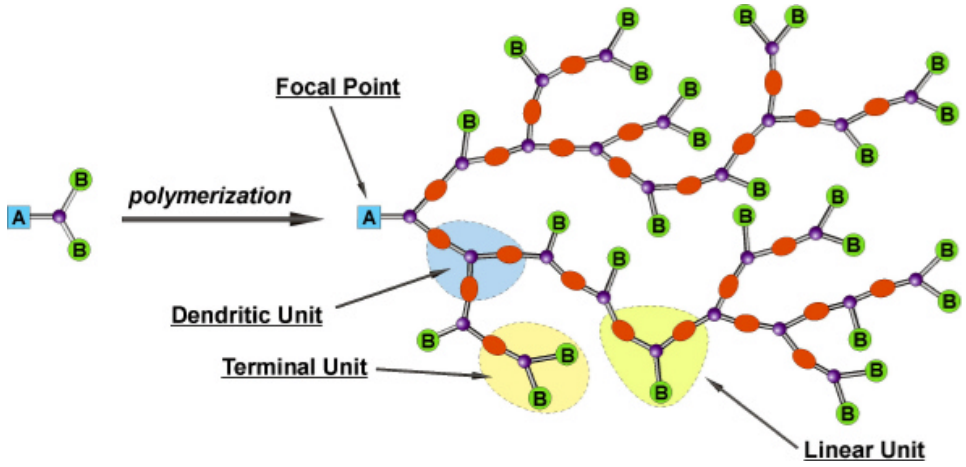


Figure 11. Schematic synthesis of HBP via polymerization of AB_2 monomers with the different structural units highlighted: dendritic (D), linear (L), and terminal (T) units. Figure reprinted from literature [29].

To compare the structure of HBPs with that of perfect dendrimers, Fréchet and co-workers introduced the "degree of branching" (DB), defined by comparing the sum of dendritic and terminal units to the total number of units in the macromolecule (Equation I).[58]
The DB is determined by NMR spectroscopy on the basis of low molecular weight model compound, that possess structures similar to linear, dendritic, and terminal units in the respective hyperbranched polymers, and is obtained by comparison of the intensity of the signals for the respective units.

$$DB\ Fréchet\ (\%) = \frac{D+T}{D+T+L}\ x\ 100 \qquad \text{(I)}$$

In 1997, Frey and co-workers proposed another expression for the degree of branching by considering the degree of polymerization (Equation II).[59] The equation does not include the terminal structural units and is therefore claimed to be more accurate than equation I for polymers with low molecular mass.

$$DB\ Frey\ (\%) = \frac{2D}{2D+T}\ x\ 100 \qquad \text{(II)}$$

Frey suggested that DB for HBPs produced by a one-pot synthesis of AB_2 monomers should be close to 0.5. One of the most important challenges in hyperbranched polymer synthesis is therefore to obtain macromolecules with narrow molecular weight distributions.

Due to the additional availability of linear (L) units, the architecture of hyperbranched macromolecules is less defined compared to perfect dendrimers. Calculations suggest that dendritic (D) units are more likely to be found in the centre of the polymer, linear (L) units are located statistically between the core and periphery of the molecule and terminal (T) units are most likely to be found at the periphery.[60,61] Nevertheless flexible dendrimer structures exhibit branch back-folding, which results in a distribution of the terminal units all over the diameter of the dendrimer. Therefore, it is possible that dendrimers and hyperbranched polymers display similar physicochemical properties due to their globular structure in solution.[62]

Additionally, hyperbranched macromolecules are characterized by the molecular weight distribution or polydispersity index (PDI or D) value (Equation III). PDI of polymers synthesized with slow monomer addition is strongly dependent on the functionality *f* of the initiator, where *f* is equal to the number of reactive groups.[63]

$$PDI = \frac{Mw}{Mn} \approx 1 + \frac{1}{f} \qquad \text{(III)}$$

Several HBPs have been developed and their properties were studied; hyperbranched polyesters, polyamides, polyphenylenes, polyurethanes, polyethers, polyamines and polyacrylates are but a few examples. These imperfectly branched macromolecules have found multiple applications as additives, coating agents, nanofoams, sensors, membranes, soluble functional supports as well as drug and gene delivery devices.[4]

Nowadays companies such as the Perstorp Group (Boltorn®, aliphatic polyesters), DSM Fine Chemicals (Hybrane®, poly(ester amides)), BASF AG (Polymin®, Lupasol®, poly(ethylene imines)), and Nanopartica GmbH (Polyglycerol, aliphatic polyethers) produce commercially available hyperbranched polymers on a large scale (Figure 12).

Hyperbranched poly(ethylene imine) (PEI), which can be synthetized by ring opening polymerization of aziridine, was the first HBP to be commercialized. Several applications have been found for PEI, derived from its polycationic character due to the presence in the structure of primary, secondary and tertiary amines. However this hyperbranched polymer is not biocompatible and can lead to long-term problems in in-vivo experiments, excluding its usage for biomedical applications.[64]

A valid alternative is certainly represented by the non-toxic, biocompatible and protein resistant dendritic polyglycerol (dPG). dPG possesses a hyperbranched, globular polyether scaffold and can be easily synthesized via ring opening multibranching polymerization (ROMB) of glycidol.[65] The

terminal hydroxyl groups, which allow for an easy functionalization, makes of the dPG a strategic scaffold for the synthesis of nanomaterials for biomedical applications.[66,67,68]

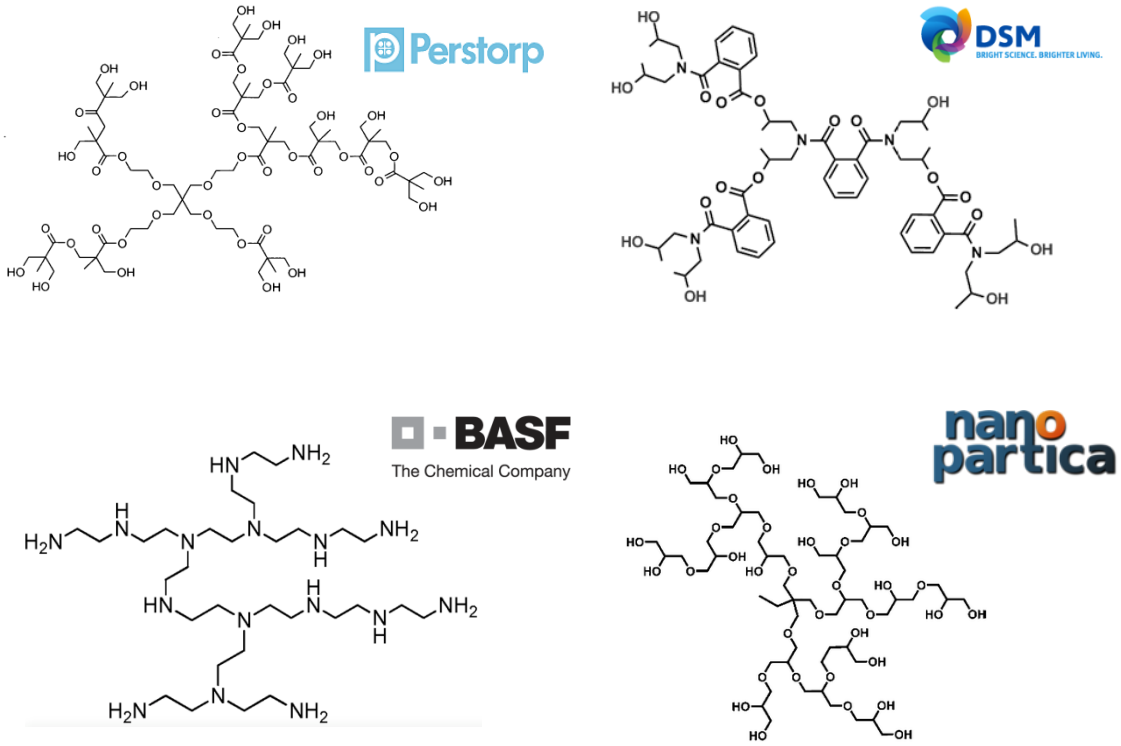


Figure 12. Structural representation of the commercially available hyperbranched polymers: Boltorn® (aliphatic polyester), Hybrane® (polyester-amide), Lupasol® (polyethylene imine) and Polyglycerol (aliphatic polyether).

1.2.3 Degradable dendritic drug delivery systems

As mentioned above, the major challenges of a drug delivery system are the targeted release of the encapsulated guest and the further facile elimination of the nanocarrier from the human body. The use of biodegradable polymeric scaffolds or biodegradable linkages can facilitate or completely solve these problems.

Almost a decade after Tomalia and Newkome *et al.* introduced well-defined and highly branched dendrimers, the first form of biodegradable dendritic polymers was simultaneously published by various research groups. [69,70]

During the last decade it was strongly proven that degradable dendritic system possesses two major advantages over the conventional non-degradable analogues: (i) multiple covalently-bound drug molecules can be site-selectively released from the nanocarrier moiety by a single cleaving step, and (ii) they can be partially or completely degraded and therefore easily eliminated from the body.[1]
Among the different degradable dendritic architectures used for drug delivery applications, it is possible to distinguish the following three classes (Figure 13):

a) Dendritic polymers with degradable backbones (pH labile, enzymatic hydrolysis, etc.)
b) Dendritic polymer cores with cleavable shells (pH environment)
c) Cleavable dendritic drug-conjugate.

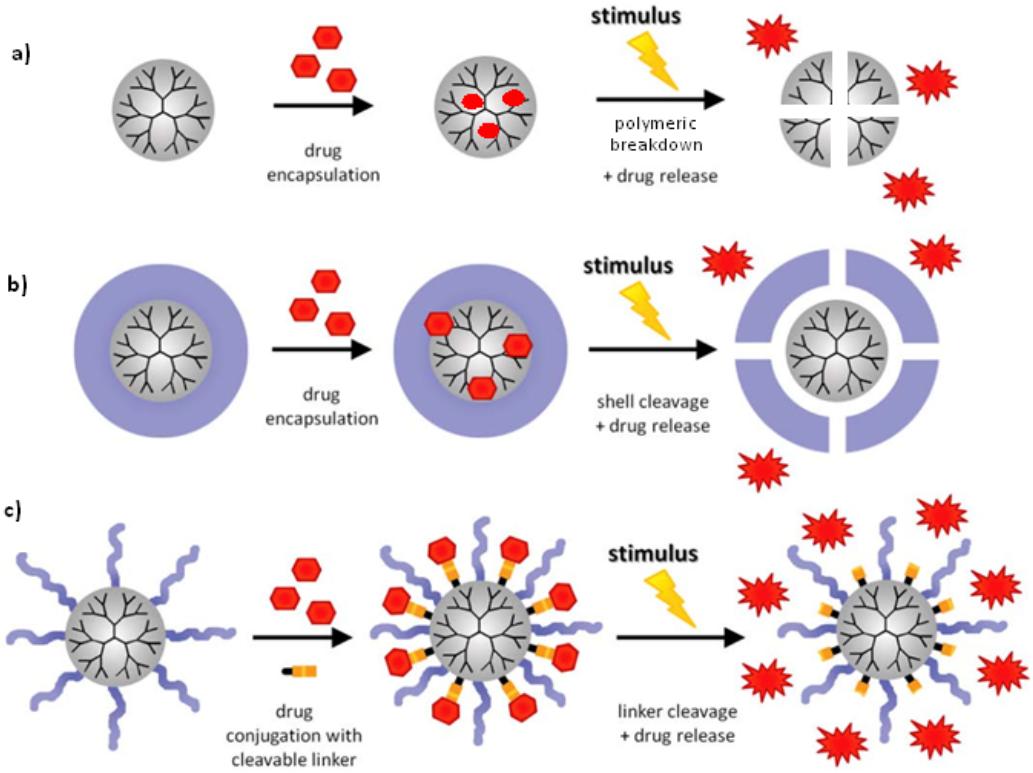


Figure 13. Release of active agents from (a) Dendritic polymers with degradable backbones, (b) dendritic core–shell particles with a cleavable shell and (c) dendritic scaffolds with attached solubilizing/stealth groups using cleavable linkers for the drug conjugation. Figure adapted from literature.[71]

In the first - and most prominent - class, the nanocarrier dendritic skeleton can be degraded or hydrolysed by external stimuli or environmental characteristics, such as acidic or alkaline pH, hydrolysis or by enzymatic degradation. The dendritic backbone can degrade in several steps in a chain reaction, releasing all of its constituent monomers and therefore the guest molecules entrapped

in the polymeric structure.
Groot *et al.* for example, reported the synthesis of a biodegradable dendrimer that has been developed to completely and rapidly dissociate into separate building blocks upon a single triggering event in the dendritic polymeric structure. They successfully achieved the release of the anticancer drug paclitaxel and proved that the by-products of their dendrimer degradation were non-cytotoxic except for the drug paclitaxel itself.[72]

It must be noted, however, that among the library of dendritic polymers employed as drug delivery systems, only a small portion of them are focused on the development of biocompatible and biodegradable hyperbranched core-based nanocarriers. These include hyperbranched polyesters, polyamide, polyester-amine, polyphosphates, polyorthoesters and polysaccharides.[73,74]

Among the several classes of degradable nanocarriers, dendritic polyesters represent the most attractive class of nanomaterials due to their biodegradability feature, even though the synthesis of these dendritic macromolecules can be challenging due to the hydrolytic susceptibility of the ester bond.[75,76] Furthermore, it was discovered that the hydrolysis rate of dendritic polyester dramatically depends on the hydrophobicity of the monomers, steric hindrance, nature of the repeating units, and the reactivity of the functional groups present in the dendritic structure.[1] In contrast, polyamide- and polyamine-based dendrimers widely used as dendritic nanocarriers, do not degrade as easily in the body and thus they may lead to a long-term accumulation *in vivo*.

In order for them to be used for biomedical applications, biodegradable dendritic polymers and their other architectures should ideally display the following characteristics: (i) nontoxic, (ii) non-immunogenic, and (iii) preferably be biocompatible.[77] Another approach widely employed is based on the possibility to chemically link - to a dendritic core molecule - an appropriate shell or directly the drug molecule via degradable or, more specifically, via stimuli responsive bonds (Figure 13 b and c). In these cases, the release involves cleavage of the linker between the carrier and the shielding shell, allowing the release of the entrapped drug (further discussed in the following chapter), or the cleavage of the linker that covalently bound the drug to the nanocarrier. These advanced nanocarriers have thus become a rather active participant in the therapeutic process than merely with an inert carrier molecule.

In Figure 14 the possible degradable and stimuli responsive linkages mostly employed in this field with the relative trigger stimuli necessary to degrade the chemical bond are displayed. [78]
The benefits of the degradable/stimuli responsive DDS are particularly important when the stimuli, to which they can degrade and release the active drug molecules, are related to the environment of the disease or to specific systemic-biochemistry parameters (e.g., specific pH, defined enzyme class, specific protein overexpression, red-ox potential).

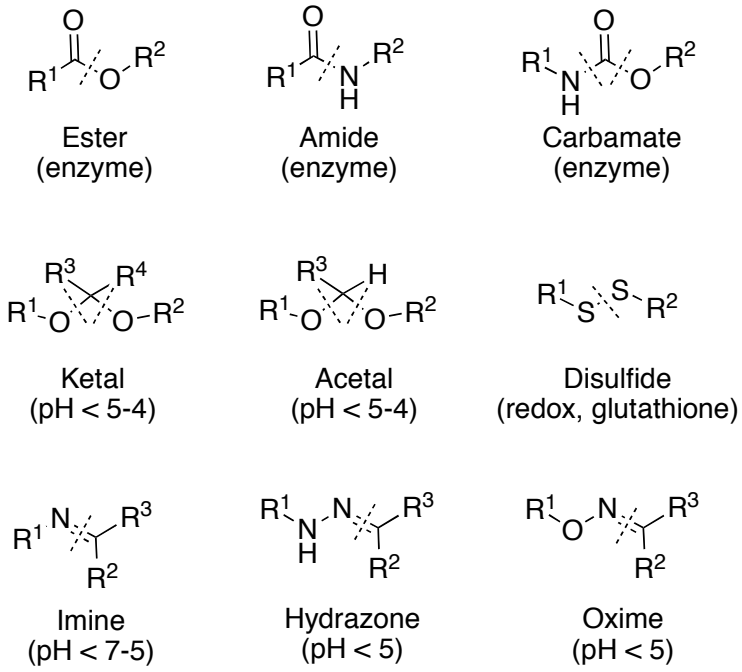


Figure 14. Cleavable linkers used for degradable and stimuli-responsive nanocarriers.

Such specificity allows the dendritic nanocarriers to selectively release their guest molecules due to a particular pathological trigger present in the diseased tissues, reducing therefore the potential side effects.[79]

For example, in some diseases, like in tumour tissues, the normal pH-gradient between extra and intracellular environment is greatly affected; the extracellular tissue can in this case be more acidic (5-6) than the systemic pH (7.4) due to reduced blood vasculature that consequently create an anaerobic environment. [80]

Calderon *et al.* have recently reported the use of a thiolated dPG scaffold for conjugation of a maleimide-prodrug of doxorubicin (DOX) or methotrexate (MTX). The use of a self-immolative para-aminobenzyloxycarbonyl spacer or the tripeptide d-Ala–Phe–Lys as protease substrate enabled them to obtain an efficient cleavage of the prodrug in the presence of cathepsin B, an enzyme overexpressed by several solid tumours. [81]

Another biological stimulus used as a trigger for the degradation of polymeric nanocarriers 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 glutathione (GSH) concentration of the considered environments. [82]

1.2.4 Core-shell and core-multishell biodegradable nanocarriers

The high functionality of the dendritic structures is not only as seen in the previous chapter, valuable for the chemical binding of drugs molecules, but as also for the grafting of different molecules in order to build an external shell and therefore tailor the characteristic of the nanocarrier.
For example, when it is necessary to increase the water solubility of hyperbranched macromolecules, methyl poly(ethylene glycol) (mPEG) is typically grafted on the external surface of the polymers, providing a hydrophilic shell around a potential hydrophobic core.[83,84,85]
PEG is a FDA approved material and is well known for its water solubility, its biocompatibility and its ability to modify the distribution of drugs, and is therefore widely used in medicine.[86,87,88] PEGylation is still considered as the golden standard to achieve the stealth effect.[89]

Currently, the most extensively-used biodegradable hyperbranched polymer is the commercially-available aliphatic polyester based on 2,2-bis(methylol) propionic acid named Boltorn® (Perstorp).[90] This partially water-soluble biodegradable polymer has been employed as a core and subsequently functionalized to increase water solubility or to change its physical characteristics in order to obtain suitable nanocarriers.
For example, Kontoyianni *et al.* reported the synthesis of a Boltorn-based H40-PEG displaying interesting unimolecular micellar properties and the capability of this system to encapsulate the anti-cancer drug paclitaxel. The solubility of the hydrophobic drug was enhanced by a factor 350 (0.28 wt.-%) in aqueous solution with a complete drug release within 10 hours.[91]
Similarly, Nyström *et al.* reported nanocarrier systems based on hydrophobic Boltorn cores (H30 and H40) with hydrophilic poly(ethylene glycol) shells (PEG5kDa and PEG10kDa). Doxorubicin (DOX) was successfully encapsulated in these biodegradable hyperbranched carriers and encapsulation efficiencies of more than 30% were achieved.[92,93]

Recently, a pH-responsive drug delivery system was developed through PEGylation of Boltorn® H40 via formation of pH-sensitive acetal linkages. Degradation experiments showed a fast hydrolysis of H40-*star*-MPEG occurred at a low pH solution (< 6), from the cleavage of the acetal linkages (Figure 15). Furthermore, it was demonstrated that DOX-loaded system could be internalized by HeLa cancer cells efficiently and the drug could be released in cytoplasm, exhibiting good anticancer efficiency.[94]

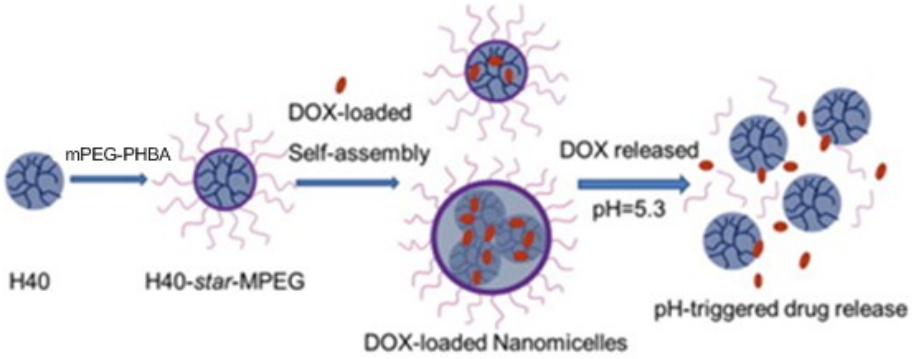


Figure 15. pH-sensitive H40-star-MPEG nanocarrier. The loading of DOX and the pH-triggered degradation of the system is presented. Figure adapted from literature [94].

Kojima *et al.* grafted PEG on the widely used PAMAM to encapsulate the drugs doxorubicin and methotrexate, proving that the amount of drug increased with increasing core and shell size.[95]

A different and cleavable hydrophilic shell was employed by D. Yan *et al.* that have developed a biodegradable core-shell system for glutathione-mediated intracellular drug delivery by employing an amphiphilic Bolton-based hyperbranched multiarm copolymer (H40-star-PLA-SS-PEP) with disulphide linkages between the hydrophobic polyester core and hydrophilic polyphosphate arms (Figure 16). The glutathione-mediated intracellular drug delivery was investigated against a Hela human cervical carcinoma cell line and it was demonstrated that this system loaded with DOX displayed a fast drug release in a reductive environment, proving the possibility of improving the antitumor efficiency of hydrophobic chemotherapeutic drugs. [96]

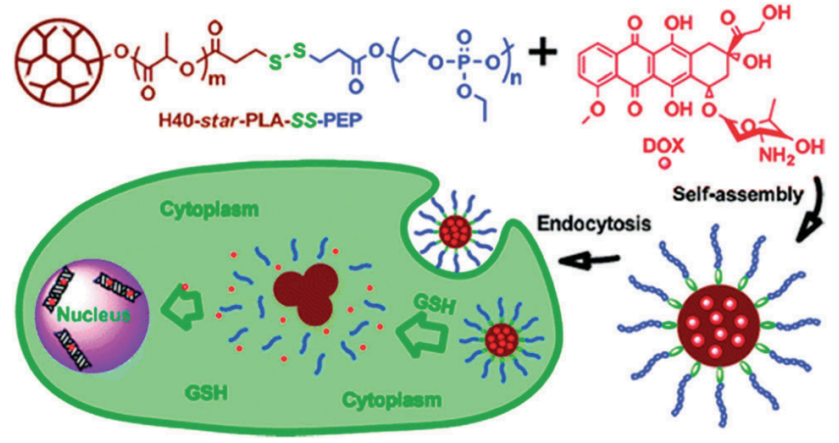


Figure 16. Cell internalization of the red-ox sensitive DOX loaded H40-star-PLA-SS-PEP nanocarrier. Figure reprinted from literature [96].

The attachment of a shell on the outer functionalized surface of hyperbranched polymers is a common procedure that allows, as reported above, not only to solubilize hydrophobic polymers but also to change its intrinsic characteristic with the aim of obtaining for examples higher drug loading capacities.

Prabaharan et al. employed H40 as a core molecule to develop a novel and more hydrophobic core-multishell system for targeted drug delivery, synthesizing H40-PLA-b-mPEG/PEG/FA. Furthermore its unimolecular micellar behaviour has been extensively studied using the anti-cancer drug doxorubicin.[97] The same core molecule was employed as a macro-initiator for the ring-opening polymerization of ε-caprolactone. PEGylation of the carboxylic external groups enables a highly efficient core-multishell drug delivery system H40-PCL-*b*-mPEG to be obtained, allowing for an efficient encapsulation of the anti-cancer drug 5-fluorouracil.[98]

Furthermore, Chen et al., incorporated folic acid to achieve tumour cell targeting property. The coupling reaction was performed between the hydroxyl group of the PEG segment and the carboxyl group of folic acid (Figure 17). Two antineoplastic drugs, 5-fluorouracil and paclitaxel, were encapsulated into the nanoparticles, and the drug releasing property and targeting of the drug-loaded nanoparticles to different cells were evaluated in vitro.[99]

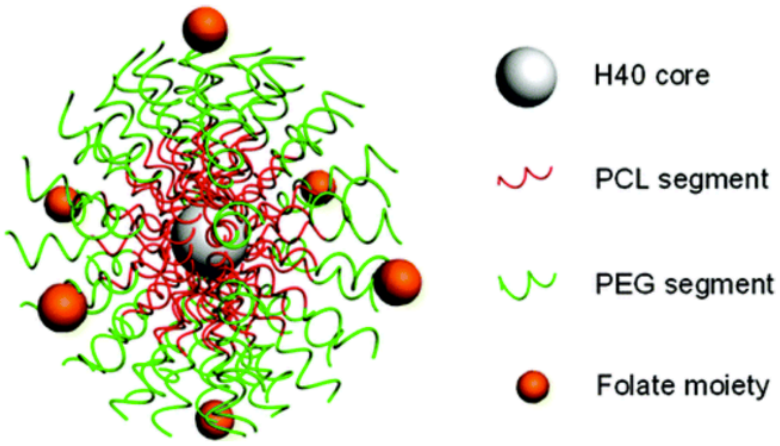


Figure 17. Schematic representation of the nanocarrier H40-PCL-b-PEG-Folate. Figure reprinted from literature [99].

Our group developed several pH-sensitive nanocarriers based on hyperbranched PEI and dPG. The functionalized surfaces of these hyperbranched polymers have been functionalized using different substituents; by attaching hydrophobic alkyl chains via acetal, ketal, or imine groups it was possible to obtain inversed unimolecular micelle architecture, while attaching hydrophilic PEG chains via an imine bond resulted in water-soluble core-shell nanocarriers.[100,101] These pH-sensitive drug delivery systems could be degraded at pH values between 5 and 7 depending on the pH-labile linker group, accompanied with the release of various guest molecules that had been encapsulated beforehand. [102,103,104,105]

By attaching to the dendritic structure a poor-hydrophilic inner shell and a water-soluble PEG outer shell it was possible to obtain a strategic polarity gradient that allowed to sensitively increase the loading of hydrophobic drug molecules. The extra shell can therefore contribute, by adding additional functions to the systems, to increase the efficiency and intrinsic characteristic of the nanocarriers. In this context, our group developed core–multishell architectures with a polarity gradient from the core to the shell (Figure 18).

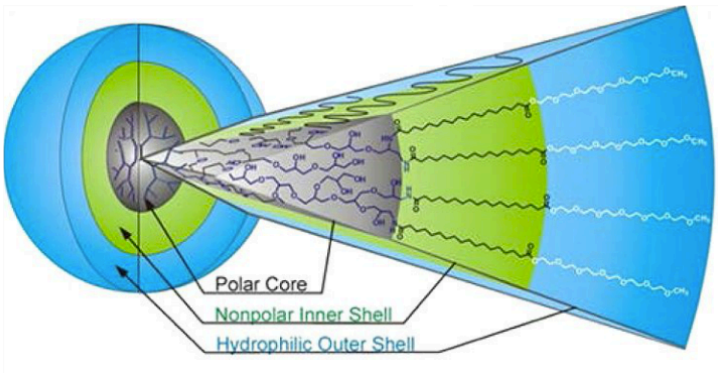


Figure 18: Schematic structure of dPG core-multishell (dPG-C18-PEG350). Figure reprinted from literature [106].

These so-called core–multishell (CMC) nanoparticles display a polar interior (biocompatible hyperbranched polyglycerol (dPG)), a hydrophobic inner shell (α–ω octadecen dicarboxylic acid), and a hydrophilic mPEG outer shell. They are soluble in water and in most organic solvents and are able to solubilise both hydrophobic and hydrophilic dyes such as Nile red and Congo red and drugs like dexamethasone and doxorubicin. Therefore these universal nanocarriers are highly efficient and suitable nanocarriers.[106,107]

The CMS nanocarriers have found various biomedical and non-biomedical applications. They have been successfully used for the vivo targeting of a F9 teratocarcinoma tumour, for the modulation of the copper level in eukaryotic cells, to efficiently stabilize metal nanoparticles (*e.g.* gold, platinum and palladium) and for different catalytic reactions. [108,109,110,111]

It was furthermore proven that CMS nanocarriers benefit from the EPR effect and are therefore able to deliver their payload more selectively into tumour tissue.[108]

In the last years CMS have been exploited as potential dermal drug delivery systems. It was found that these systems are indeed able to significantly enhance the skin penetration of different guest molecules.[112,113]

1.3 Nanocarriers for dermal drug delivery

The skin is the vastest human organ and due to its fascinating characteristics, this organ covers many complex and vital functions. The major role is to provide a protective barrier of the body from the external environment. The minimization of water loss and the prevention of invasion by external threats such as microbes, toxic agents, viruses, irradiation, and particulate matter, into the organism, are certainly the most predominant ones. The skin in fact possesses several defensive mechanisms which prevent most nanoparticles or nanosize materials from penetrating the skin, unless the tissue is disrupted, based on physical, metabolic, immunological, or UV-protective barriers.[114]

In recent years the scientific community has had a common thought; once the characteristics and the defence mechanisms are completely understood, the skin can be used for non-invasive dermal or transdermal delivery of drugs. Dermal and transdermal drug delivery are seen as a safe and efficient way to deliver active agents, but the efficient overcoming of the skin barrier still remains a great challenge. [115]

By taking an overview in the field of dermal drug delivery, it can be seen that all drugs currently administered through the skin exhibit similar characteristics: low molecular mass (below 500 Da), marked lipophilicity, and low dose concentration. The delivery of bigger, or/and more hydrophilic drugs molecules into the skin thus remains a great challenge.[116]

Several types of nanoparticles have been developed to overcome the skin barrier, but most of them failed to be introduced onto the market often due to limited stability (e.g. liposomes) or due to an unknown safety profile (e.g. several polymer nanoparticles). [117,118]

Besides several nano- and microparticles that have been exploited for this purpose, dendrimer-mediated dermal drug delivery has obtained, in recent years, increasing interest. [119,120]

As shown in figure 19, three different layers can be detected in the skin: the dermis (D), the viable epidermis (VE) and the stratum corneum (SC). The dermis is the lower layer of the skin and it is located between the viable epidermis and the subcutaneous tissues. It is formed by a variety of cell types, nerves, collagen, elastic fibres, blood vessels, and a lymphatic system, which are held together by connective tissue. It also contains macro-receptors that provide the sense of touch and thermo-receptors that provide the sense of heat. [121]

On top of the dermis, separated by a basement membrane layer, lies the viable epidermis that is an avascular tissue mainly composed of stratified keratinocytes (95%) surrounded by an extracellular lipid matrix. Keratinocytes, anuclear coneocytes, act as the body's major barrier against an invasion by external threats by preventing pathogens from entering. [121] Several enzymes are present in the keratinocytes and are fundamental for several biological processes. Compared to the liver the total

metabolic capacity of the epidermis is low; nevertheless, due to the vast dimension of this organ, cutaneous biotransformation proved to be of considerable relevance. [122]

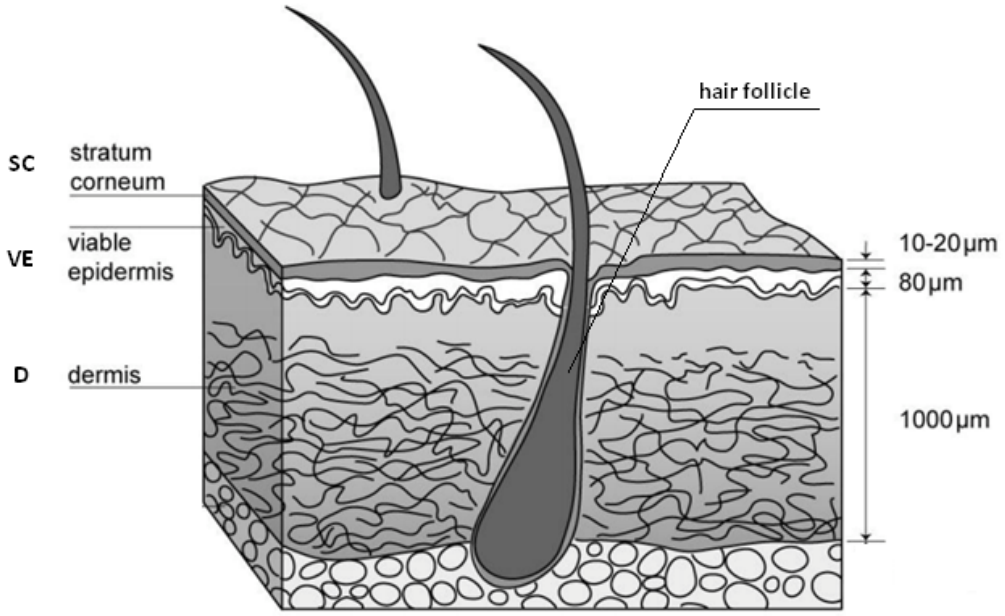


Figure 19. Schematic representation of the different skin layers; stratum corneum (SC), viable epidermis (VE) and dermis (D). Figure adapted from literature.[123]

The outermost layer of the epidermis, the stratum corneum "horny layer", is the most important barrier of the skin. The SC consists of 15-20 layers of flattened dead cells (corneocytes) filled with aggregated keratin and reinforced by the cornified envelope; corneocytes are the "bricks" in the mortar of the stratum corneum lipid matrix.[118,124] The stratum corneum is composed of three lipid components: ceramides, cholesterol and fatty acids.[125] Diffusion through the stratum corneum is therefore for most substances the rate-limiting step of skin permeation.

Depending on the therapeutic aim, the drug needs to be delivered either into the epidermis and/or into the dermis; three possible approaches of enhanced cutaneous penetration have been presented to explain the mode of action of dendritic nanoparticles: release modifier, penetration enhancer, or trap in hair follicles. In the first case, the polymeric nanocarrier loaded with the active substance is applied on the skin, allowing for the obtention of a "customized" release of *i.e.* a slow and constant release of the drug encapsulated or to simply increase the drug dissolution. In the second pathway, the drug-loaded polymeric nanocarrier is capable of penetrating the deeper layers of the skin by perturbing the lipid bilayers impairing the stratum corneum. Finally, the dendritic carriers might help to target the hair follicles. Despite the fact that only a small surface of the human skin is covered with hair, the

deep invagination and high vascularization of the hair follicles makes this route an interesting approach to obtain an efficient dermal drug delivery. [126]

Although several dendritic nanoparticles for cutaneous drug delivery have been developed,[127] systems based on dendritic polyamidoamine (PAMAM) and polyglycerol (PG) are certainly the most studied ones.

Several studies have reported the skin penetration and permeation enhancing effects of PAMAM dendrimers. The effects of the surface properties and of the particle size are the primary focus and therefore the loaded drug cutaneous uptake was studied in rodent or porcine skin *ex vivo*. It has been demonstrated that cationic PAMAM dendrimers increased drug permeation more than neutral and anionic PAMAM nanoparticles. The intense interactions of the positively charged particles with the bilayers of stratum corneum lipids are considered the driving force of the penetration process.[128] Furthermore, it has been reported that the size of PAMAM dendrimers also influences their skin penetration. As discovered in skin permeation studies, generation 4-PAMAM dendrimers penetrate deeper skin layers when compared with the analogue generations 3 and 5. [129,130,131]

The aforementioned dPG-based core-multishell (CMS) nanocarriers (Figure 18) have been studied as dermal drug delivery systems and it was proven that they efficiently enhance the skin penetration of the hydrophilic dye Rhodamine B and the hydrophobic dye Nile red. [132,133] The penetration of both guests could be significantly increased compared to a conventional base-cream application (Figure 20). The CMS nanocarriers were even superior to solid lipid nanoparticles (SLN), which are considered the golden standard for dermal drug delivery.

Furthermore, it was found that CMS nanocarriers are non-cytotoxic against human keratinocytes cells, non-irritant and do not interfere with skin cell migration, promoting the CMS nanocarriers as excellent candidates for cutaneous drug delivery application. [134,135]

Due to the interesting results obtained, more recently, CMS nanocarriers were employed to enhance the skin penetration of different peptides.[136] Cell-Penetrating Peptides (CPPs), Low Molecular Weight Protamine (LMWP) and penetratin were labelled with a fluorescent dye (Lissamine Rhodamine B) and loaded into the CMS nanocarriers. After application on human skin of unloaded labelled peptides and CMS-loaded labelled peptides it was found that even in this case, the presence of the polymeric nanocarrier significantly enhances the penetration of the peptides into the different layers of the human skin (Figure 21).

It was shown that the CMS nonocarriers do not penetrate the epidermis, but support the transport of the dye through the stratum corneum.[137]

However, it was suggested that dendrimers interact with the skin surface and influence lipid organization of the stratum corenum which may lead to enhanced skin penetration of drugs.[138,139]

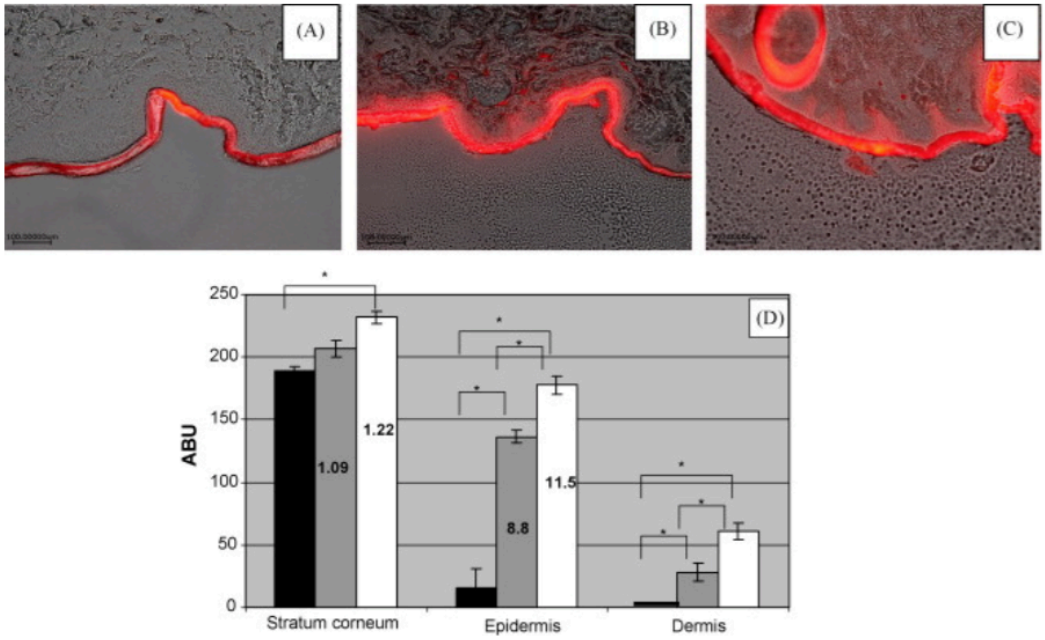


Figure 20. Rhodamine B penetration into pig skin: staining of porcine skin following the application of 0.004% rhodamine B loaded cream (A), SLN (B), and CMS nanocarriers (C) for 6 h. (D) The arbitrary pixel brightness values (ABU) obtained by fluorescence picture analysis (cream, black columns; SLN, grey columns; CMS nanocarriers, white columns). The inserted numbers display the respective enhancement of penetration.[132]

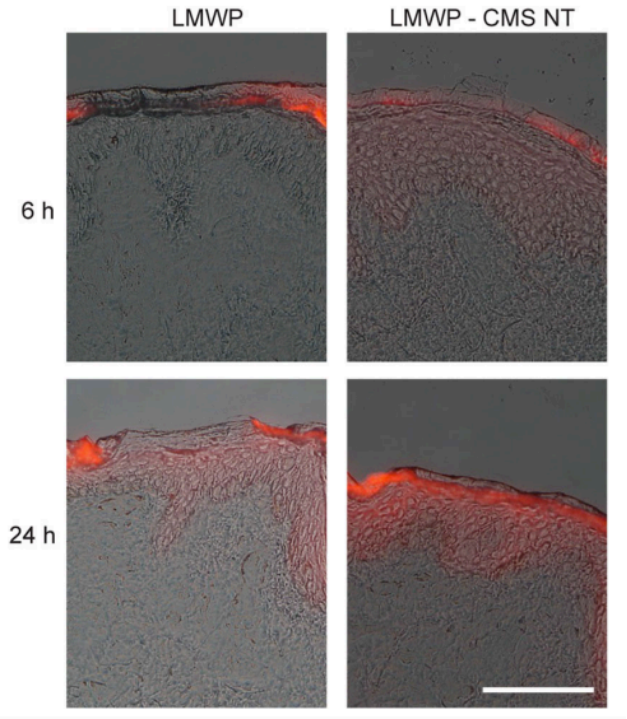


Figure 21. Enhancement of LMWP penetration in the presence and absence of CMS nanotransporters in skin. Fluorescence microscopy at 6 h and 24 h post application of 0.01 mM Lissamine Rhodamine B labelled LMWP in the absence or presence of CMS nanotransporters (CMS NT).[136]

2. Scientific Goals

Polymeric drug delivery systems (DDS) can significantly enhance the performance of several active agents for the treatment of different diseases. To optimize the drug loading capacity and the targeted release efficiency these systems have to be specifically tailored in order to boost their capability as potential nanocarriers.

The goal of this work is initially focussed on the development of new biodegradable hyperbranched polymers. As previously mentioned, the easy clearance of the nanocarrier from the body after releasing the cargo is of primary importance in the field of DDS. The polymeric skeleton can, in these cases, be degraded or hydrolysed by external stimuli or environmental characteristics, for example, acidic or alkaline pH, hydrolysis or by enzymatic degradation. The dendritic backbone commonly degrades in several steps in a chain reaction, releasing all of its constituent monomers and therefore the guests molecules entrapped in the polymeric structure. The intrinsic characteristics of the hyperbranched polymers (*i.e.* hydrophobicity, nature of the chemical linkers) can influence the biodegradation rate and thus the release profile of the encapsulated guest molecules. Furthermore, the optimization of these characteristics is of primary importance in order to achieve high drug-loading capacity. For all the above-mentioned reasons, a set of hyperbranched biodegradable polymers will be developed and their properties will be studied in order to obtain important information regarding their characteristics and biodegradation behaviour.

The properties of the hyperbranched polymers synthesized will be modified by the addition of an eternal shell to their globular surfaces. Binding hydrophilic or amphiphilic chains to the periphery of highly hydrophobic hyperbranched polymers allows to increase the water-solubility and to obtain a

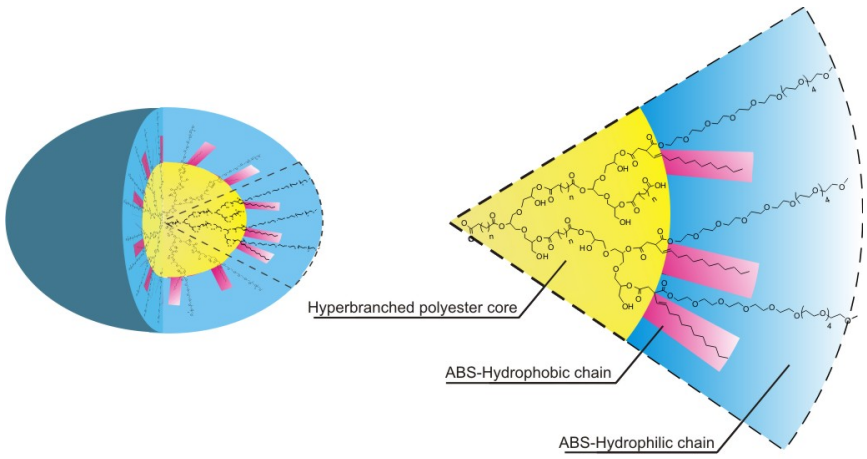


Figure 22. Schematic representation of a core - amphiphilic branched shell nanocarrier.

so-called core-shell architecture, characterized by a lipophilic internal core and a hydrophilic external shell.
The development of a new typology of shell that can promote the loading of hydrophobic guest molecules into the nanocarrier whilst ensuring its water solubility thus became of fundamental importance for this project.

The last objective of the research will be focussed on the testing of the novel macromolecules developed as potential drug delivery systems. Several hydrophobic or poorly water-soluble dye and drugs molecules (i.e. pyrene, Nile-red, Doxorubicin, and Dexamethasone) are selected and will be used as guests for the in vitro tests.
Particular attention will be paid to understanding the release processes. Release tests will be carried out under physiological conditions and by employing a hydrolytic enzyme like CAL-B that should catalyse the degradation of the polymeric backbone with the corresponding release of the entrapped guest molecules.

Finally, in order to promote the core-shell macromolecules as potential dermal drug delivery systems, tests to evaluate penetration of guest molecules though the different layers of the human skin will be carried out in collaboration with the group of Prof. Hedtrich.

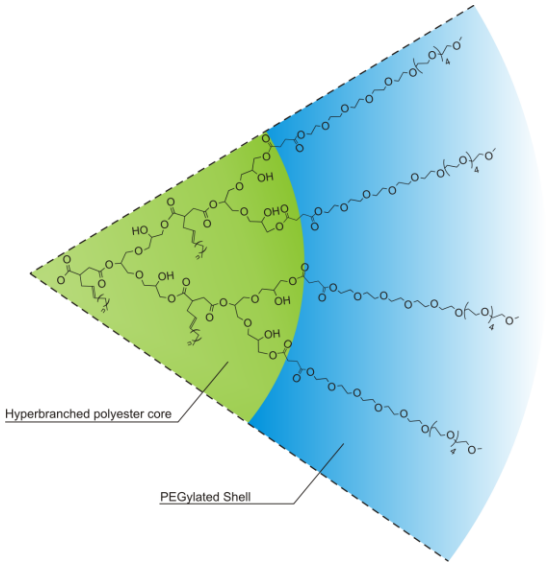
3. Publications and Manuscripts

3.1 Core-shell nanocarriers based on PEGylated hydrophobic hyperbranched polyesters.

This chapter was published in: S. Stefani, P. Servin, S. Sharma and R. Haag, *European Polymer Journal,* **2016**, 80, 158-168. http://dx.doi.org/10.1016/j.eurpolymj.2016.05.005

Contribution of the authors:

The complete synthesis of the nanocarriers, the encapsulation studies of pyrene, DXM, FNS and further enzymatic release studies were carried out by the author. Furthermore, the author personally carried out the aggregation studies via DLS and the preparation of the manuscript.



ABSTRACT

Two strategic core-shell biodegradable hyperbranched polyesters have been developed as drug delivery system (DDS) to enhance the solubility of highly hydrophobic molecules. Herein we describe an optimized synthesis of triglycerol based hydrophobic polyesters and the subsequent PEGylation to obtain two water-soluble derivatives characterized by different hydrophobicity. Pyrene has been tested as references to evaluate the potential drug transport capability and the critical micelle concentration (CMC) of the systems. Experiments showed that in one case it was possible to load up to 1.9 wt% of pyrene with an almost complete release within 16 days under enzymatic conditions. Furthermore the hydrophobic drugs Dexamethason (DXM) and Finesteride (FNS) were also efficiently encapsulated into the novel nanocarriers.

3.2 Triglycerol-based hyperbranched polyesters with an amphiphilic branched shell as novel biodegradable drug delivery systems

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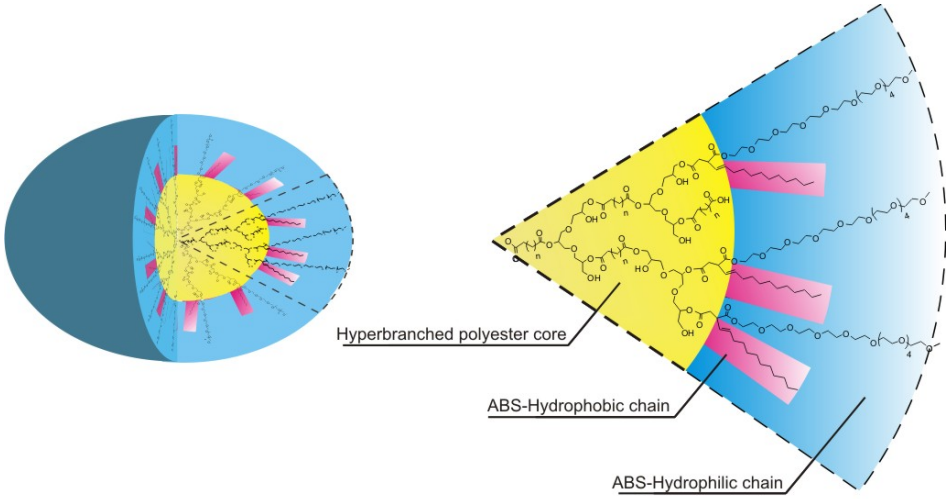
Contribution of the authors:

The complete synthesis of the nanocarriers, the encapsulation studies of pyrene, DOX, DXM and the further enzymatic release studies were carried out by the author. Dr. Christoph Böttcher carried out the cryo-TEM measurements. Furthermore, the author personally carried out the biodegradation studies and the preparation of the manuscript.



ABSTRACT

The synthesis of biodegradable triglycerol-based hyperbranched polyesters (HBPEs), characterized by different hydrophobicity, has been optimized and described. A new amphiphilic branched shell (ABS) was developed; PEGylated amphiphilic chains were attached to the external corona of the HBPEs, with the aim of enhancing the encapsulation efficiency of hydrophobic drugs while additionally encouraging the solubilization of the HBPEs in aqueous media. Pyrene was tested as a template to evaluate potential drug transport capacity and to obtain information about the microenvironment and binding sites of the drug carriers. Experimental tests have shown the excellent capabilities of the aforementioned systems as drug delivery systems (DDS); it was possible to load up to 4.1 wt% of pyrene, evenly released from the system, within 9 days in the presence of Candida Antarctica lipase B (CALB). Subsequently the anticancer drug doxorubicin and the anti-inflammatory steroidal drug dexamethasone were efficiently encapsulated in the ABS-nanocarriers.

3.3 Hyperbranched glycerol-based core-amphiphilic branched shell nanotransporters for dermal drug delivery

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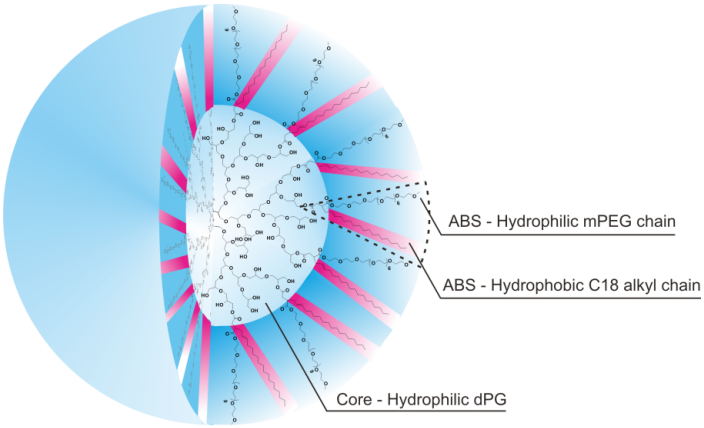
Contribution of the authors:

The complete synthesis of the nanocarriers and the encapsulation studies of pyrene, DXM, FNX were been carried out by the author. Furthermore, the author personally carried out the aggregation behaviour studies via DLS and the preparation of the manuscript.
Dr. Jose Luis Cuellar Camacho, Falko Neumann and Stefan Hönzke carried out the AFM measurements, cytotoxicity experiments and the skin penetration evaluation respectively.



ABSTRACT

A novel amphiphilic branched shell (ABS) obtained upon reaction of octadecen-1-yl succinic anhydride and methyl polyethylenglycol ($mPEG_{500}$) is herein presented. Through chemical attachment to the functionalized external surfaces of three different glycerol-based hyperbranched polymers (HBP), a set of novel core-ABS nanocarriers that were tested as possible candidates for dermal delivery of active substances was obtained. Pyrene has been studied as a model to evaluate the potential drug transport capability and the critical micelle concentration (CMC) of these amphiphilic systems. Subsequently, the anti-inflammatory steroid drug Dexamethasone and Finasteride, a drug widely used to prevent prostate cancer and androgenic alopecia, were also efficiently loaded into the nanocarriers. In order to promote these systems as possible candidates for dermal drug delivery, tests to evaluate the cytotoxicity on human keratinocyte cells and the penetration of the encapsulated molecules into the different layers of human skin samples were carried out.

4. Summary

This work presented the synthesis and the development of a set of novel biodegradable nanocarriers characterized by a core-shell and core-branched shell architecture. Their properties and aggregation behaviour in solution were studied and experiments to prove their biodegradability and their potential capabilities as drug delivery systems were successfully carried out.

In the first section of this work, two novel hyperbranched polyesters characterized by a highly hydrophobic core were synthesized by polymerizing the triglycerol with two different alkenyl-succinic anhydride (ASA) derivatives. The unreacted accessible hydroxyl groups still present after polymerization were reacted with the free carboxylic acid of the $mPEG_{500}$-succinate in order to obtain the desired water-soluble core-shell nanocarriers. The novel carriers were used to efficiently encapsulate hydrophobic dyes like pyrene and drugs such as dexamethasone and finasteride.

Furthermore it was possible to obtain, under enzymatic conditions, a slow and almost linear pyrene release profile; it was possible to release over 80% or the dye encapsulated within 16 days. Interestingly, no significant release was observed under physiological conditions in absence of the enzyme.

The core-shell nanocarriers form aggregates of different sizes when dissolved in aqueous media; their aggregation behaviours were studied by DLS analysis deducting that the hydrophobicity of the polymeric core is certainly the driving force for the formation of these architectures and proved its importance in order to significantly increase the loading capacity of hydrophobic guest molecules.

In the second section of the work, the optimized synthesis of three new biodegradable hyperbranched polyesters characterized by different hydrophobicity, produced by reacting the triglycerol with different α–ω dicarboxylic acids (succinic acid, adipic acid and dodecanedioic acid) is described.

Biodegradation tests in the presence of the enzyme CALB showed that it was possible, in one case, to hydrolyze over 50% of the ester linkages within 9 days.

A novel amphiphilic branched shell was developed and synthetized by reacting an ASA bearing a C12 alkenyl substituent with an $mPEG_{500}$ chain. Further attachment, via an ester bond, to the accessible hydroxyl groups of the HBPEs enable the formation of the core-amphiphilic branched shell (ABS) architecture. The properties of the core–AB-shell nanocarriers characterized by different polymeric cores were studied and initial preliminary tests to ensure their candidature as drug delivery systems were carried out using poorly water-soluble drugs like DOX and DXM and dyes like pyrene.

The amphiphilic branched shell displayed a double important role on the development of the novel nanocarriers. It is increasing the loading capacity of the nanocarriers characterized by a polar polymeric core as well as helping to solubilize in aqueous media the nanocarrier having a hydrophobic polymeric core.

The nanocarrier characterized by a highly hydrophobic core proved to be the best candidate for drug delivery applications; it was possible to increase the solubility of pyrene in water by a factor of over 300. Furthermore, a pseudo-linear delivery profile in the presence of enzyme (CALB) allowed a release of over 60% of the encapsulated pyrene in 9 days.

Proven the high performances of the novel core-ABS nanocarriers, the work was further extended; to boost their capabilities, a more hydrophobic ABS was developed using a C18ASA in place of the previously employed C12ASA. The further attachment of the C18-ABS to the external periphery of the aforementioned hyperbranched polyesters and to the dPG enable the formation of even better performing nanocarriers.

In one case, comparing two nanocarriers having the same polymeric core, but different amphiphilic shell, it was possible to denote an average increase of 40% in loading capacity of hydrophobic guest molecules when the more hydrophobic amphilphilic shell was employed.

The cytotoxicity of the systems was also tested on human keratinocyte cells; all the systems displayed no cytotoxicity at a concentration of 0.05 mg/mL.

Furthermore, initial skin penetration tests showed that the core-ABshell carriers are able to efficiently transport Nile red as a model therapeutic agent through this complex biological barrier. It was possible to increase - approximately eleven-fold - the transport of the guest molecule into the viable epidermis, compared to the ability of a classical base cream.

5. Zusammenfassung

Die vorliegende Arbeit beschreibt die Synthese und Entwicklung einer Reihe neuartiger biologisch abbaubarer Nanocarrier (Nanotransporter), deren Charakteristikum eine Kern-Schale-Architektur ist. Im Verlauf der Arbeit wurde die Schale durch eine innovative amphiphile und verzweigte Schale (ABS) substituiert. Die Eigenschaften und das Aggregationsverhalten der Nanocarrier in Lösungen wurden untersucht und es wurden erfolgreich Experimente hinsichtlich ihrer biologischen Abbaubarkeit und ihrer Eignung als Drug-Delivery-Systeme durchgeführt.

Im ersten Abschnitt der Arbeit wurden zwei neuartige hochverzweigte Polyester (HBPE) synthetisiert, die einen stark hydrophoben Kern besitzen. Um einen wasserlöslichen Kern-Schale-Nanocarrier zu erhalten wurde zunächst Triglycerol mit zwei verschiedenen alkylierten Bernsteinsäureanhydrid-Derivaten (ASA) polymerisiert. Die nach der Polymerisation noch verfügbaren und nicht reagierten Hydroxylgruppen wurden anschließend mit den freien Carbonsäuren des $mPEG_{500}$-Succinimid umgesetzt. Die neuartigen Nanocarrier wurden zur effektiven Verkapselung hydrophober Farbstoffe wie Pyren sowie von Wirkstoffen wie Dexamethason oder Finasterid verwendet.
Ferner war es möglich unter enzymatischen Bedingungen eine langsame und fast lineare Freisetzung von Pyren zu erhalten; es konnten Freisetzungsraten innerhalb von 16 Tagen von über 80% des verkapselten Farbstoffs erreicht werden. Interessanterweise konnte bei Abwesenheit des Enzyms unter physiologischen Bedingungen keine signifikante Freisetzung beobachtet werden.
Die Kern-Schale-Nanocarrier bilden Aggregate unterschiedlicher Größe wenn sie in wässrigen Medien gelöst werden; das Aggregationsverhalten wurde untersucht und mittels DLS – Analyse konnte als Triebkraft dieser Architekturen die Hydrophobizität der polymeren Kerne identifiziert werden was die Bedeutung der signifikanten Steigerung der Ladungskapazität der hydrophoben Gastmoleküle bestätigt.

Im zweiten Abschnitt der Arbeit wird die optimierte Synthese von drei neuen biologisch abbaubaren HBPE beschrieben die durch verschiedene Hydrophobizitäten charakterisiert sind. Die Herstellung erfolgt durch Reaktion von Triglycerol mit verschiedenen α-ϖ Dicarbonsäuren (Bernsteinsäure, Adipinsäure und Dodecandisäure).
Untersuchungen zur biologischen Abbaubarkeit durch das Enzym CALB zeigten, dass es in einem Fall möglich war über 50% der Esterbindungen innerhalb von 9 Tagen zu hydrolysieren.
Durch Reaktion einer C12-alkylsubstituierten ASA (C12ASA) mit einer $mPEG_{500}$-Kette konnte eine neue amphiphile und verzweigte Schale (ABS) entwickelt und synthetisiert werden. Das Verknüpfen der freien Hydroxylgruppen der HBPE durch eine Esterbindung führte zur Bildung der Kern-ABS-Architektur. Die Eigenschaften von Kern-ABS-Nanocarriern mit unterschiedlichen polymeren Kernen

wurden untersucht. In ersten Versuchen wurde mittels schwer wasserlöslicher Wirkstoffe wie DOX und DXM sowie Farbstoffen wie Pyren ihre Eignung als Drug-Delivery-System geprüft.
Die ABS-Architektur zeigt zwei wichtige Eigenschaften der entwickelten neuartigen Nanocarrier. In Verbindung mit einem polaren polymeren Kern wird die Ladungskapazität der Nanocarrier gesteigert, zum anderen verbessert sie die Löslichkeit in wässrigen Medien der Nanocarrier mit hydrophoben polymeren Kern.
Als am besten geeignete Kandidaten für Drug-Delivery-Anwendungen wurden die Nanocarrier mit einem stark hydrophoben Kern identifiziert. Die Löslichkeit von Pyren in Wasser konnte bei ihnen um mehr als Faktor 300 gesteigert werden. Des Weiteren erreichte die pseudo-lineare Freisetzung des verkapselten Pyrens über 60% innerhalb von 9 Tagen in Gegenwart des Enzyms CALB.

Nach Überprüfung des guten Struktur-Eigenschafts-Profils der neuartigen Kern-ABS-Nanocarrier wurde die Arbeit ausgeweitet. Um die Aufnahmefähigkeit von Wirkstoffen weiter zu steigern, wurde durch Verwendung eines C18ASA anstelle des zuvor verwendeten C12ASA eine noch hydrophobere ABS-Architektur entwickelt. Durch Anfügung des C18ABS an die Oberfläche der oben beschrieben HBPE und an dPG konnten Nanocarrier mit noch besserer Transportkapazität geschaffen werden.
Der Vergleich zweier Nanocarrier mit gleichem polymeren Kern aber verschiedenen amphiphilen Schalen zeigte in einem Fall für den Nanocarrier mit stärkerer hydrophober amphiphiler Schale eine durchschnittliche Steigerung von 40% Ladungskapazität hydrophober Gastmoleküle. Auch die Zytotoxizität der Systeme wurde an humanen Keratinozytzellen untersucht; alle getesteten Systeme zeigten keinerlei Zytotoxizität bei Konzentrationen von 0,05 mg/mL.
Des Weiteren zeigten erste Hautpenetrationstests, dass die Kern-Schale-Nanocarrier in der Lage sind den Farbstoff Nilrot, ein Modell für therapeutische Stoffe, effektiv durch die komplexe biologische Barriere zu transportieren. Im Vergleich zur Transportfähigkeit gewöhnlicher Cremes konnten etwa elfmal mehr Gastmoleküle in die obere Epidermis gebracht werden.

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7. Curriculum Vitae

For reasons of data protection, the Curriculum vitae is not published in the online version.