3 LITERATURE SURVEY

3.1 Polymeric Materials for Advanced Drug Delivery

Besides biopolymers, synthetic macromolecules play a central role in drug delivery technology. Macromolecular delivery devices with specific physical and biological properties have generated continuous interest in novel polymer synthesis, in both academic and industrial[III] environments.

Since this thesis in particular deals with dendrimers as drug delivery devices for anticancer-therapeutics, it is neither the aim of this survey to summarize the entire context of polymeric drug delivery systems nor to review the history of dendrimers. The focus is on the influence of biocompatibility, site-specific drug delivery aspects, and mechanisms for a controlled release, on the design of suitable carriers, and especially, on applications of dendrimers in medicinal chemistry, in particular anticancer-therapeutics.

3.1.1 Toxicity and Immunogenicity of Dendrimers

The synthetic concepts to solubilize polymeric carriers in an aqueous medium have a considerable impact on their toxicity. Dendrimers are either solubilized as poly electrolytes (cationic or anionic) or by employing OEGs or poly ethylene glycol chains (PEGs).

Generally, polycationic structures can destabilize cell membranes to the point of cell lysis.[40] Typical representatives of this class of carriers are PAMAM-(Starburst^{TM}), DAE-, DAB-, PPI-, and polylysine-dendrimers (Fig. 4).

Comparative studies on different cell-lines have shown a generation-dependent toxicity, with dendrimers of higher generation and with more free amines being more toxic.[41-43] These results are in accordance with the haematotoxicity studies by Duncan and coworkers.[44] They conclude that the haemolytic effect of PAMAM dendrimers on rat blood cells is increasing with the increasing generation of the dendrimers. However, PAMAM dendrimers have lower toxicity than their linear

[III] According to a SciFinder research: in total 705 patents are related to dendrimers; therefrom 74 related to “drug delivery” and 18 to “cancer therapy”.

analogues.[43] This can be explained by the lower adherence of the less flexible amines of a globular dendrimer to cellular surfaces. Furthermore, the toxicity is influenced by the degree of substitution on the amines, with primary amines being more toxic than secondary or tertiary.[43, 45]

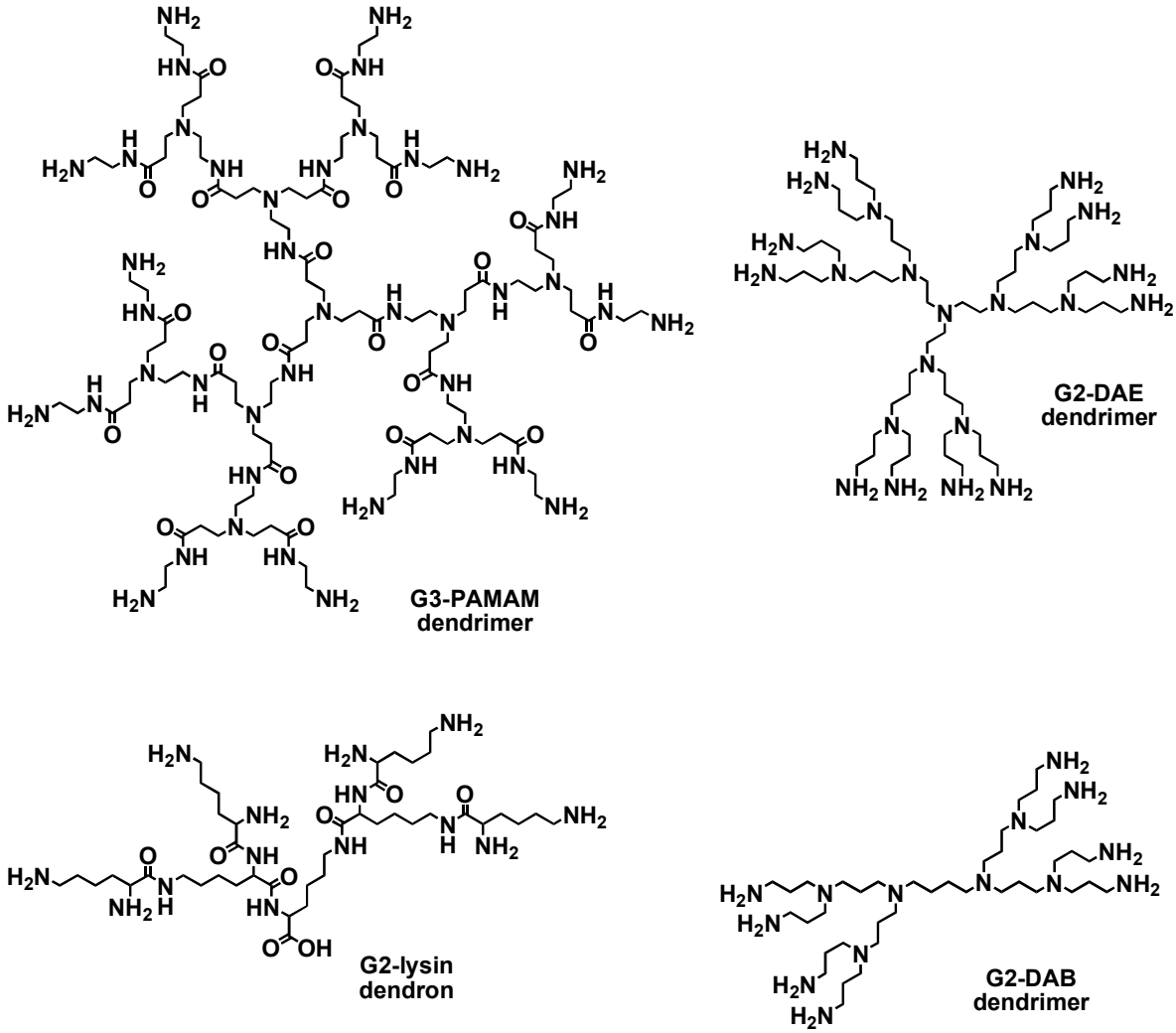


Fig. 4. Dendritic polycations.

On the contrary, dendrimers carrying ethylene-diamine moieties seem to be nontoxic, regardless of their generation. Initial systematic studies on unmodified PAMAMs show weak immunogenicity for dendrimers up to generation seven.[41, 46] In summary, polycationic amino-terminated carriers are not optimal candidates in the context of drug delivery because most of these toxic structures damage the cell membranes and cause cell lysis.

Typically, compounds with anionic surface groups are less toxic than their cationic counterpart. Examples for this class of carriers are derivatives of PAMAM-(Starburst™), DAE-, DAB-, and PPI-dendrimers with carboxylic acid termination (Fig.

5, under physiological conditions, the acids are deprotonated and can be regarded as anions). In comparison, they have a significantly lower toxicity than the amino-terminated species. Since the generation of the dendrimers in these comparative studies is the same, it can be concluded that the dendritic backbone plays a minor role regarding the observed toxicity.[42] Furthermore, low-generation PAMAM-dendrimers with anionic surface groups neither showed haematotoxicity nor cytotoxicity at concentrations of 2mg/ml.[44] Thus it seems, that carriers with a polyanionic surface are better candidates for drug delivery purposes than their cationic counterparts.

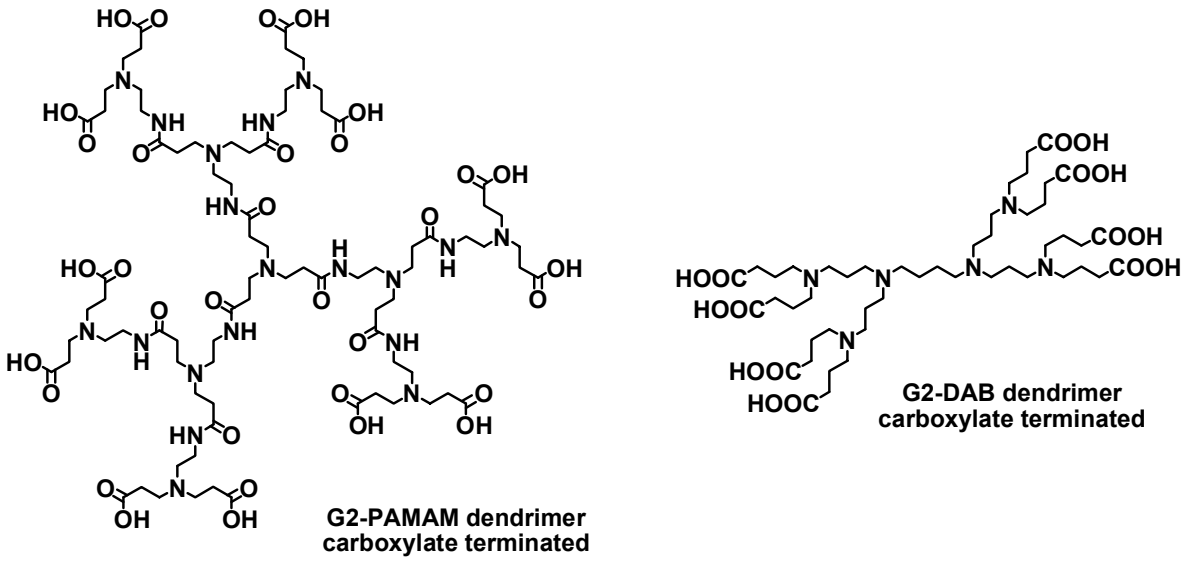


Fig. 5. Dendritic polyanions.

It is quite remarkable that additives can lead to a significant decrease in the toxicity of poly electrolytes. The addition of fetal calf serum to PAMAM dendrimers, which were partially modified with the fluorophore Oregon Green, reduced the cytotoxicity towards human cancer cells (HeLa) in comparison to the modified dendrimers alone. Additionally, DNA complexed PAMAMs up to generation three do not possess any significant cytotoxicity *in vitro*.[47, 48] These results are also confirmed by a different study in which PPIs with amino groups in the periphery are likewise less toxic when used as DNA complexes.[49] Consequently, a non-covalent coating of a dendrimer can possibly shield the cationic polyelectrolyte and reduce its toxicity. However, studies on DNA-G5 PAMAM complexes showed the same or even higher toxicity compared to the non-modified amino-terminated PAMAMs.[50] This increased toxicity is attributed to both a cellular stress upon transfection with large amounts of DNA (3μg/mL) leading to apoptosis and to remaining positively charged amines on the surface of

the incomplete, wrapped dendrimer.[51] However, the *in vitro* studies are not representative for an analogous *in vivo* essay. Only a few systematic studies on the *in vivo* toxicity of dendritic molecules are published. Surprisingly, generation five PAMAMs do not appear to be toxic upon injection with 10 mg/kg in animals.[41, 52] In a different study, where mice are treated with mixtures of PAMAMs and ovalbumin, neither weight loss, granuloma formation or haemolysis, nor inflammation are observed.[46]

Another approach to make dendrimers soluble in water was accomplished using OEGs and PEGs. These solubilizers are easy to handle in organic solvents due to their amphiphilic character. They are used for surface modification of poly electrolytes and as solubilizers for other dendritic scaffolds. Dendrons are connected to linear or star-like PEGs (Fig. 6).[53-55] The linkage is either between the focal points of dendrons or on the periphery of dendrimers. The PEGs are either directly attached to a dendritic surface or they are grown by anionic polymerization with ethylene oxide.

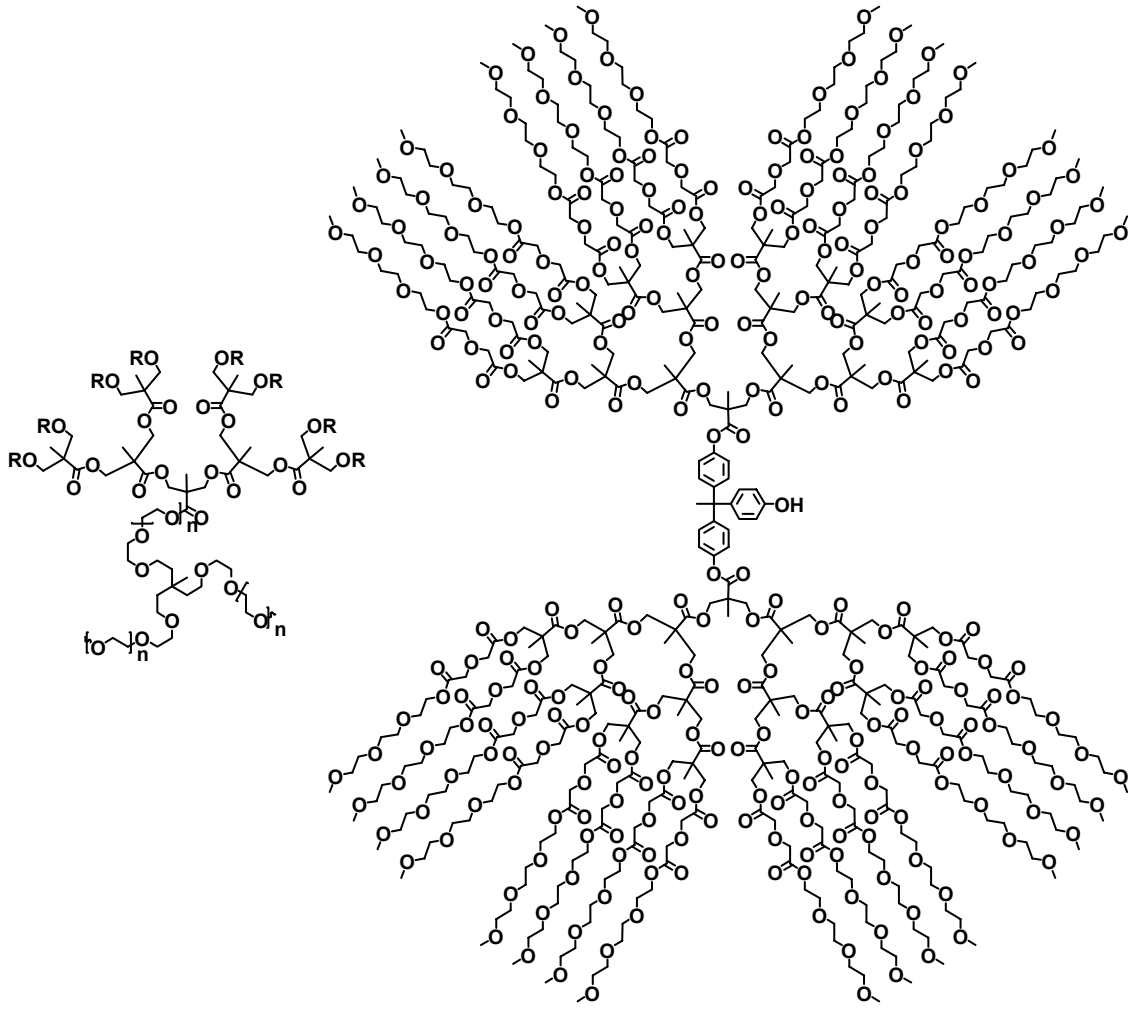


Fig. 6. Dendrimers with OEGs and PEGs.

The cytotoxicity of PEGylated PAMAMs is reduced significantly.[42] Such dendrimers with a polyester scaffold are non-toxic, both *in vitro* and *in vivo*.[54, 55] At very high concentrations (40 mg/mL) cell growth is inhibited *in vitro*, but no cell death is observed. Furthermore, no acute or long term toxicity is indicated upon injection in mice. PEGylated PAMAMs have a reduced immunogenicity in comparison to unmodified PAMAMs. PEGs increase hydrophilicity and generate a highly hydrated surface with a low disturbing effect on the physiological environment.[56] Since toxicity essays do not indicate intrinsic toxicity of PEGs or PEGyated dendrimers, these solubilizers can be regarded as exceptional candidates to enhance the solubility of a carrier system.

3.1.2 Site-Specific Drug Delivery Aspects

Preferentially, a drug delivery system should target the release of a drug to a precise site of action. A specific distributional control can be extremely valuable to diminish the typical drawbacks of classical medication. An increase in the efficiency of therapeutics is especially important when side effects are too severe and limit or prevent further treatment, or when the natural distribution does not accumulate enough drug molecules to the site of action.[7]

Tumor targeting can be accomplished in two different ways. The EPR effect as a passive, site-specific delivery was already mentioned and will not be discussed in more detail. On the other hand, it is possible to selectively address a certain binding-site of a receptor or antigen which is overexpressed on the surface of tumor cells.

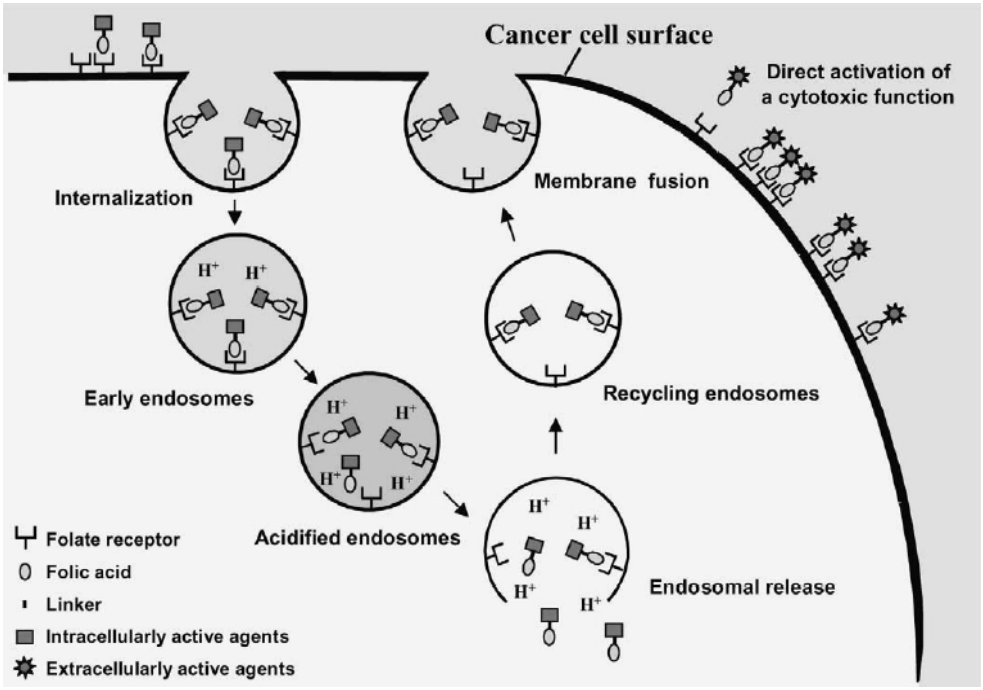
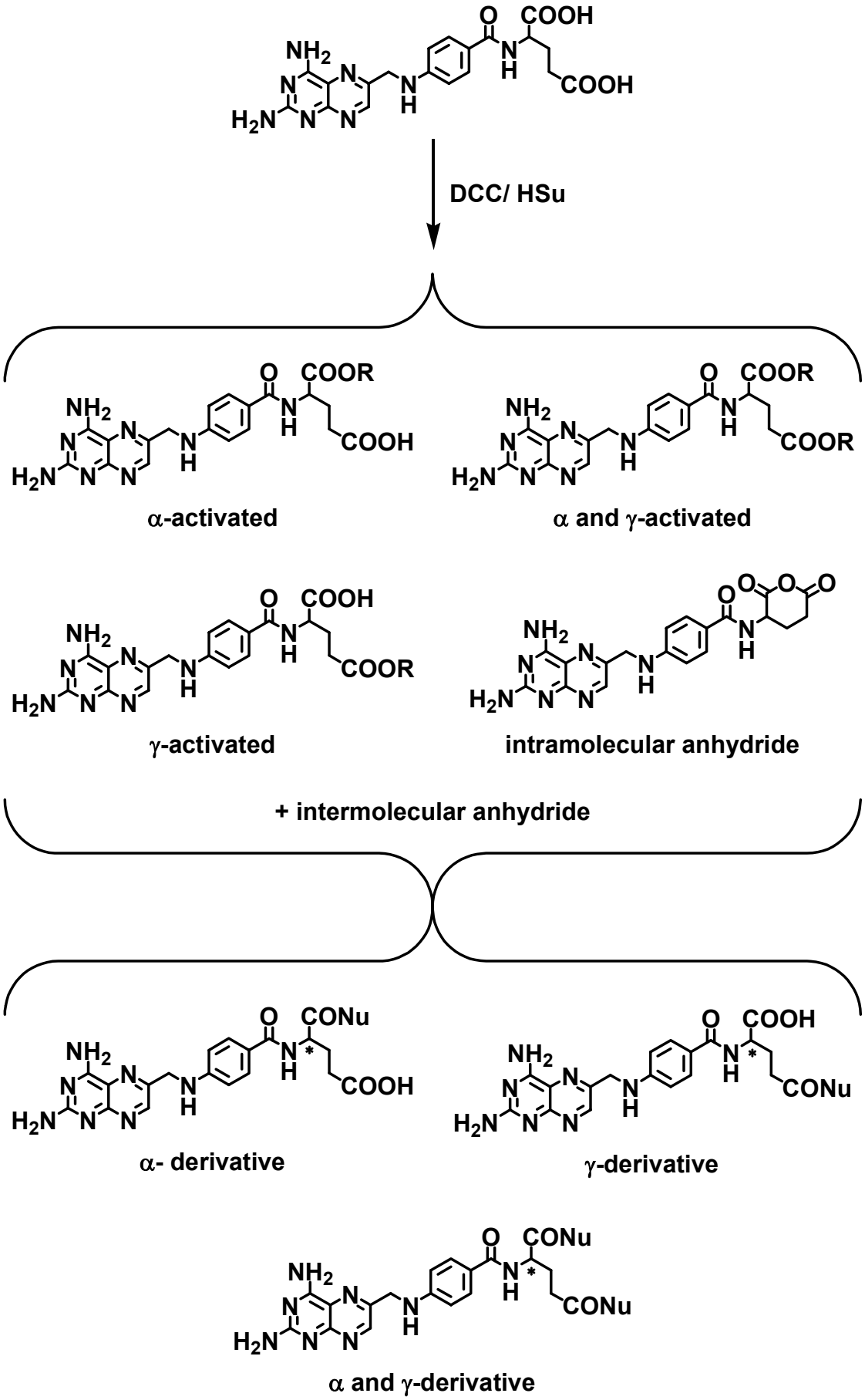


Fig. 7. Folate mediated delivery of drugs via endocytosis.[57]

An example for this kind of active targeting is the specific interaction with the folate binding protein (FBP), a highly selective tumor marker over-expressed in greater than 90% of ovarian carcinomas. Two membrane-bound isoforms of the folate receptor (FR) have been identified in humans, designated α and β. These isoforms bind an oxidized form of folate, folic acid (FA).[58] The receptor binding properties of FA are retained when derivatized via its γ-carboxyl, and weakened when derivatized via its α-carboxyl.

Even though the precise mechanism of FR transport of FA into cells remains uncertain, it is clear that folate conjugates are taken up by cells via ligand activated endocytosis (Fig. 7).[57] However, the regioselectivity of the activation process and its implication for the composition of the resulting drug-conjugates is a major problem. The product profile for both the activation and the coupling step is quite complex. Besides α- and γ- mono-activated derivatives, a standard peptide coupling protocol using *N*,*N*'-dicyclohexyl-carbodiimide (DCC) and *N*-hydroxysuccinimide (HSu) yields various by-products like *bis*-activated derivatives and anhydrides. In consequence, several undesirable products are formed in the reaction with nucleophiles and, furthermore, couplings at the α-carbonyl proceed with complete racemization (Scheme 5).[59] However, under optimized conditions it is possible to drive the reaction to completion, with an average γ-connectivity of 65%.[60]



Scheme 5. Product profile for the activation of folic acid.

3.1.3 Controlled Release of Drugs

Since there are ways to deliver a drug specifically to a tumor cell, the controlled release of drugs from the conjugate in an active form is the next important consideration for the design of polymeric conjugates. Three main concepts can be defined for a selective liberation of drugs from a carrier (Fig. 8).

In the first concept, drugs are directly attached to a biodegradable carrier. This approach has already been discussed in the introduction and will not be considered in more detail.

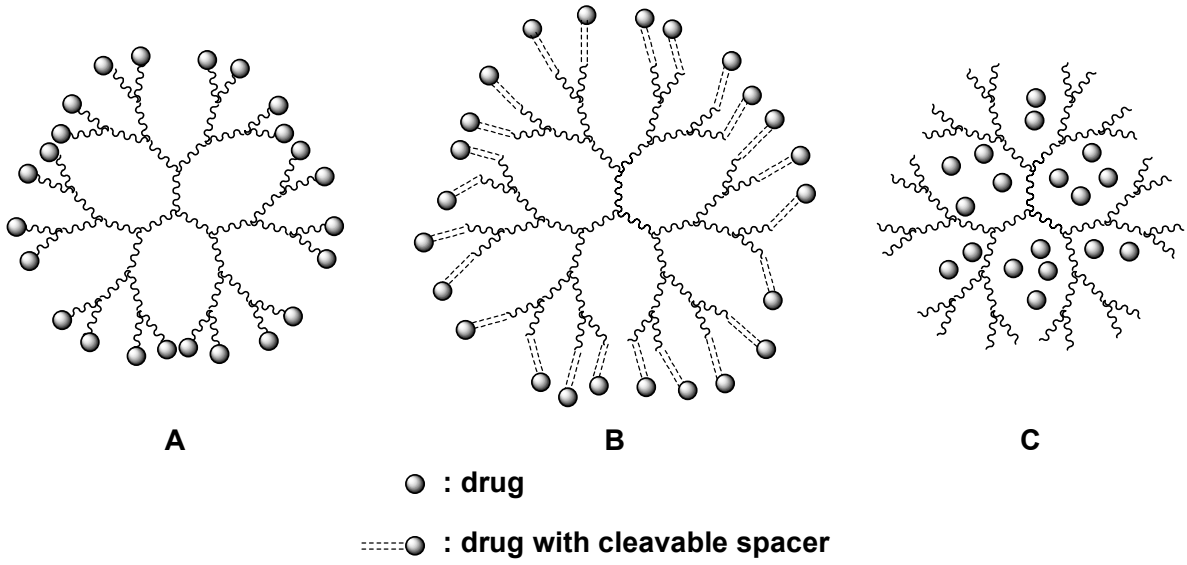


Fig. 8. Illustration of different release concepts.

The second concept is based on the introduction of a labile spacer between carrier and drug. These linkages use the low pH value in the tumor environment. The spacer is a covalent connection, stable in healthy tissue, and degraded in more acidic endosomal or lysosomal compartments. A range of acid-responsive interfaces, based on ortho esters,[61] acetals,[62] ketals,[62] and hydrazones,[55] are available. The main difference of these reagents is their rate of hydrolysis in intracellular compartments. Their major disadvantage is the rate-dependence, where the rate of cleavage is proportional to the pH-value. A single pH unit changes the rate of degradation by a 10-fold difference due to the logarithmic definition. This means that the hydrolysis will always be a compromise between a slow rate in healthy tissue with a high pH and a fast rate in tumor tissue with low pH value. Alternatively, the labile interface can employ tumor specific enzymes for its intracellular cleavage into two smaller peptides or, in the case of a *di*-peptide, into the corresponding amino acids. Suitable peptides

typically consist of 2 to 4 amino acids and are easily accessible via solid-phase synthesis. However, the binding efficiency of the cleavage side to the active side of the enzyme has to overcome the sterical constraints of a polymeric carrier.

The third concept does not use a covalent attachment of the drug to the carrier and is based on the intrinsic structure of dendrimers. The “dendritic box” is one of the first examples for a “host guest” system which works as an endo-receptor and benefits from the cavities of a dendrimer. The “host guest” principle can arise from both hydrophobic and polar interactions, depending on the structure of the dendrimer. The association between guests of different sizes and dendrimers is attributed to both electrostatic interactions and the sterical demand of the bulky surface-groups.[63-65]

3.2 Platinum Compounds in Anticancer-Therapy

Throughout examination of the effect of electric fields on the growth of *Escherichia coli* cells, a biological activity of platinum compounds was uncovered. Cell division was stopped and induced filamentous growth in the bacteria.[66-68] The most effective compound formed by the reaction of platinum from the electrode with ammonium chloride, namely cisplatin, has been known since 1845,[69] but not until 1970 its tumor activity in mice was discovered.[67, 68, 70] The compound was approved by the FDA in 1979 and the cure rate for testicular cancer is greater than 90% when tumors are diagnosed in an early stage (Fig. 9).[71] Other kinds of malignancies, including ovarian, cervical, head and neck, and non-small-cell lung cancer are treated with cisplatin as well.[72-75]

The exact biological activity for cisplatin and the precise mechanism has remained elusive. However, it is generally accepted that DNA platination is the ultimate event in the mechanism of action of platinum anticancer drugs.[76] The administration of the drug is done by intravenous injection. In the blood-stream cisplatin encounters a relatively high concentration of chloride ions (100 mM) which suppresses the hydrolysis to the aquated forms *cis*-$[Pt(NH_3)_2Cl(OH_2)]^+$ and *cis*-$[Pt(NH_3)_2(OH_2)_2]^{2+}$, respectively. Since the uptake is not carrier-mediated, the drug enters cells by passive diffusion.[77] Inside the cell the lower chloride concentration (~2-30 mM) facilitates hydrolysis to the activated, positively charged form, which is attracted to negatively charged DNA via electrostatic interactions.[78] Since the rate of dissociation of a nucleotide platinum binding compared to the rate-limiting hydrolysis is extremely

slow, the binding process has been assigned as a kinetic rather than thermodynamically driven process.[79]

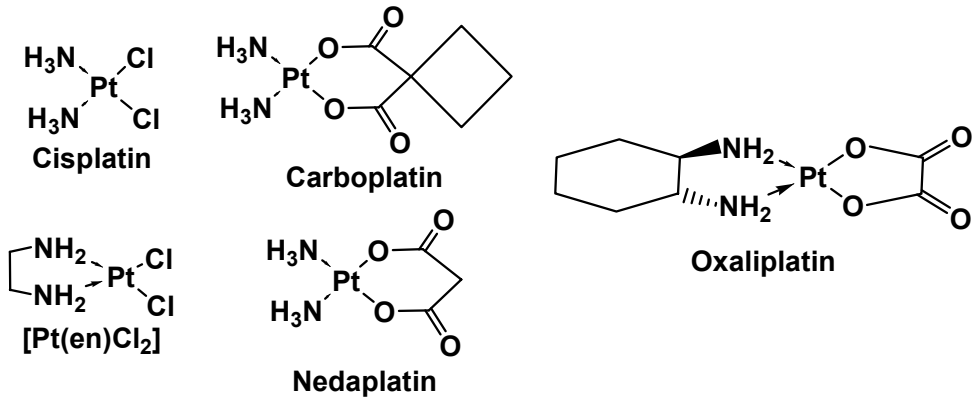


Fig. 9. Examples of well-known platinum compounds with biological activity.

The most accessible and reactive nucleophilic sites for platinum binding to DNA are the N7 atoms of the imidazole rings of the purin bases adenine and guanine in the major groove of the double helix.[80] These complexations lead to the formation of various structurally different adducts. The precoordinated monofunctional DNA adducts further react to produce distorted, kink-like interstrand or intrastrand cross-links, which then inhibit replication and transcription.[81] However, binding of cisplatin to non-DNA targets is a major problem. Only 5-10% of covalently bound, cell-associated drug is found in the DNA, whereas 75-85% bind to proteins.[82, 83] Phospholipids and phosphatidylserine of the cell membrane can compete in the binding process and lower the accumulation of the drug in the cell.[84] In the cytoplasm cellular components with soft nucleophilic sites, such as thiol containing peptides and proteins and RNA react with cisplatin.[85] Cisplatin is not the only platinum compound with a biological activity used for anticancer-therapeutics. Other examples such as carboplatin and oxaliplatin may lead to the conclusion that the *cis*-geometry is a strict structural requirement for the action of the drug. Newer platinum complexes have now been developed which in a few cases deviate significantly from the classical ones. A more general interpretation concludes that Pt-complexes of a certain size and shape, with a certain polarity, capable of weak, moderate, or strong binding to biomolecules, may show anticancer activity.[86, 87]

The structure of the ligands is very important for the properties of the platinum compounds. Since the strength of the platinum binding decreases from primary or secondary amines to carboxylic acids, which are weaker leaving groups than

chlorides, these parameters have a high impact on the stability and efficiency of the drug. Additionally, ligands can target drugs site-specifically to tumors.[88-92]

These drugs still have serious drawbacks, including low water solubility, small tumor cell selectivity, and the inherent or acquired resistance seen in many tumors.[2]

3.3 Dendrimers in Medicinal Chemistry

Since the high potential of dendrimers arises from their versatile, well-defined structure and the possibility to control both peripheral and internal functionalities, these molecules are very useful. They have attracted considerable interest for many different research areas,[65, 93-122] and, in particular, they are explored and used for life-science applications.[16, 53, 104, 123-131] As dendrimers are monodisperse macromolecules with a large number of tailorable motifs on the surface, they are very valuable for therapeutic and diagnostic purposes.

3.3.1 Dendrimers in Gene-Therapy

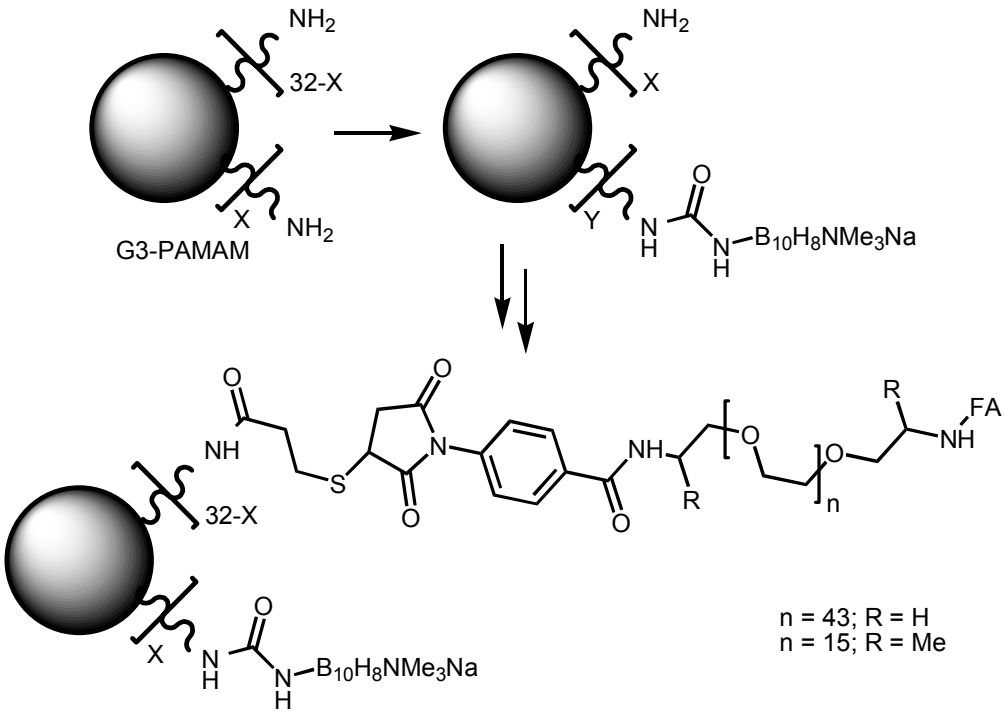
Since genetic defects can be corrected by transferring active genes into damaged cells, systems such as viruses, linear cationic lipids and cationic oligopeptides have been employed as transfection tools. However, the use of viruses is associated with risks. Most linear polycationic vectors are cytotoxic and have a low delivery efficiency.[128, 132] Dendrimers with primary or secondary amines, which are positively charged under physiological conditions, can establish a “host guest” binding to the anionic phosphate moieties in the DNA backbone due to an acid-base interaction. Haensler and Szoka were the first to study the gene-delivery properties of PAMAM dendrimers.[128] They partially functionalized the PAMAMs with a cysteine-containing peptide analogue which can destabilize membranes of lipids and allow formation of a stable complex with negatively charged DNA. The best transfection activity was obtained for a generation five PAMAM when an excess of terminal amines to nucleotides was used. The efficiency was 10 to 100 times higher compared to lipid based reagents.[129, 133, 134] Purified monodisperse PAMAMs still showed low levels of transfection, while structurally imperfect and partially degraded dendrimers are better suited for such purposes.[133-135] These degradation studies led to the conclusion that the transfection activity is more dependent on the positive net charge of the complex and on its flexibility than on its molecular weight.[134] The transfection kit SuperFekt™ is based on these results. It is commercially available and widely used in molecular

and cellbiological research.[IV] Saltzman and coworkers conjugated PAMAMs with PEG prior to complexation with plasmid DNA.[132] The system had an improved flexibility, less electrostatic interactions with the DNA and in particular proved to have a very low toxicity. The toxicity was dependent on the generation of the dendrimer, with increased toxicity for a higher generation. An interesting example for a phosphorus containing dendritic gene-delivery device was developed by Majoral and coworkers.[136] These dendrimers have an extraordinary stability in water over a wide pH range and their intrinsic scaffold is highly flexible and porous. The dendrimers with a grafted, positively charged surface showed significant transfection ability towards the luciferase gene within 3T3 cells.

3.3.2 Dendrimers in Neutron-Capture-Therapy

Neutron capture therapy (NCT) is a biomedical therapy. A nontoxic substance is preferentially delivered to the tumor, followed by its conversion or activation into a radiotoxic substance. The activation process occurs after neutron capture of low-energy neutrons. Since two isotopes, ^{10}B and ^{157}Gd, have a significantly large capture cross-section they are of major interest. The neutron capture event for ^{10}B results in the transformation to the exited ^{11}B nuclei. The fission of ^{11}B yields highly energetic α-particles and $^{7}Li^{3+}$ ions. These charged particles create ionization tracks along their trajectories which trigger cellular damage or cell death.[137] The neutron capture cross-section for ^{157}Gd is more than 66 times larger than that for ^{10}B.[138] Upon neutron capture ^{157}Gd generates Auger and internal conversion electrons which are strongly toxic in cells.[139]

[IV] SuperFect™ is a product of Quiagen GmbH (Hilden, Germany).



Scheme 6. PAMAM dendrimer with targeting function for neutron capture therapy.

Since both candidates have a low bioavailability, and a large amount of nuclei in a cell is needed to cause cellular damage, several research groups employed dendrimers as carriers for neutron capture nuclei.[138, 140-144] A very nice example for the site-specific delivery of boronated PAMAM dendrimers was reported by Shukla and coworkers (Scheme 6).[145] They randomly derivatized a third generation PAMAM dendrimer with a shortage of *closo*-$Na(CH_3)_3NB_{10}H_8NCO$ decaborate cluster in different ratios. The remaining free amines were covered with a PEG-folic acid building block. Biodistribution studies with these conjugates resulted in selective tumor uptake, but also in high hepatic and renal uptake.

3.3.3 Dendrimers in Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a modern diagnostic technique based on proton NMR which is used to visualize, for example, organs and blood vessels. A well-defined inhomogeneous magnetic field has an influence on the relaxation time of H_20-protons in tissue. Since signal intensity depends on both the relaxation of the adjacent tissue and the shift of the pattern relative to the neighboring tissue, the obtained resonances correlate to the micro environment and display soft tissue.[127] The resolution of these measurements can be increased by employing paramagnetic metal ions (Gd^{3+}-complexes), which modify the spin-lattice relaxation and suppress

the fluctuation of bordering magnetic fields. Since contrast agents with low molecular weight diffuse to extravascular tissue and consequently eliminate quickly from the blood circuit, it is reasonable to use macromolecular carriers that can delay this process. Dendrimers are advantageous on linear and branched polymers, because the motif of a contrast-agent can be multiplied by their large number of peripheral groups and, in particular, they are less flexible. Wiener and coworkers reported PAMAM based Gd^{3+} chelates. The generations 2 and 6 which possess 12 and 192 reactive terminal amines were conjugated to a chelating ligand through a thiourea linkage. [146, 147]

Another study on PAMAM conjugates found a proportional dependence of the relaxation on the molecular weight and the generation of the dendrimer. The increasing interest of the pharmaceutical industry on such systems illustrates their potential. A team of the Schering AG obtained the most promising results with their "gadomer 17" (Fig. 10). This MRI contrast agent is a second generation polylysine dendrimer with a trimesic acid core moiety. The periphery is covered with 24 Gd^{3+}-ions in a chelating ligand system.[127, 148-150]

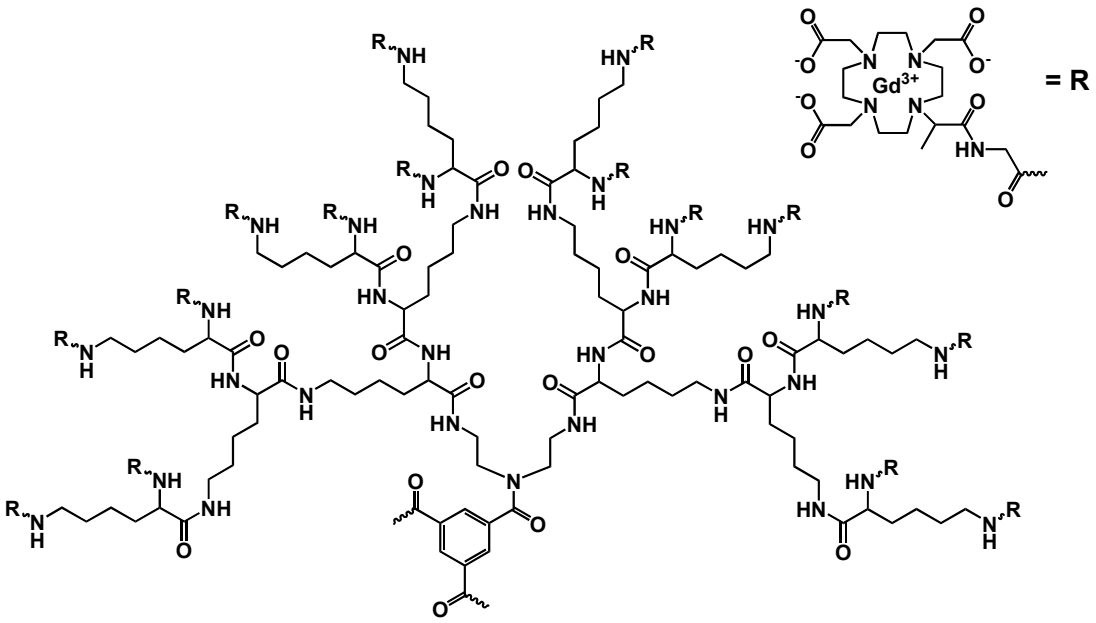


Fig. 10. Schering's "gadomer 17".

3.3.4 Dendrimers in Anticancer-Therapy

The reference describing a conjugate of a dendrimer and an anticancer drug was published in 1999 by Zhuo and coworkers.[151] They synthesized PAMAM dendrimers with a 1,4,7,10-tetraazacyclododecane core moiety and acetylated around 50% of

the terminal amines. The remaining primary amines were alkylated with a fluorouracil derivative. The hydrolysis of these conjugates in a phosphate buffer solution (pH 7.4) at 37°C released free fluorouracil. A little later, Fréchet and coworkers came up with another idea to use dendrimers as drug delivery devices for anticancer-therapeutics.[53, 152, 153] They employed poly(aryl ether) dendrimers which were semi-coated with short PEGs to achieve solubility in water. The remaining surface groups were then used to incorporate cholesterol, L-phenylalanine, or L-tryptophan, respectively. A few months later, the same group published a different approach.[61] The periphery of another poly(aryl ether) dendrimer with 16 surface groups was covered with either folic acid or methotrexate. With an average load capacity of 78% these conjugates were soluble in aqueous medium above pH 7.4.

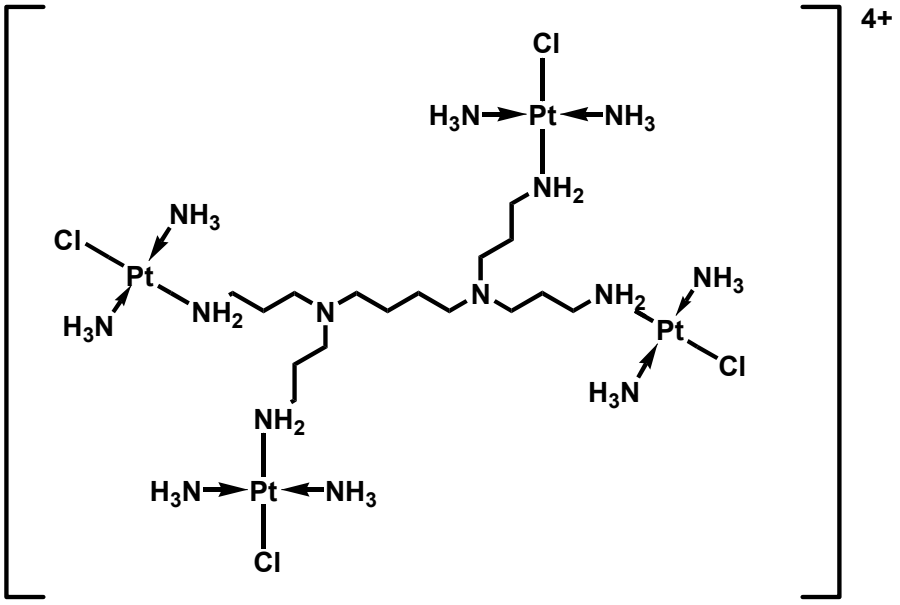


Fig. 11. PPI dendrimer with *tetra*-nuclear transplatin complex.

Still in 1999, Reedijk and coworkers reported the synthesis of a conjugate consisting of a PPI dendrimer and transplatin.[154] Their concept aims at overcoming cisplatin resistance and is based on previously published dinuclear transplatin compounds, which were connected by flexible diamine linkers (Fig. 11). The reaction with guanosine 5'-monophosphate confirmed that these conjugates can still bind up to four nucleobases. Another early approach towards a dendrimer-platinate was published by Duncan and coworkers.[155] Their preclinical study included the synthesis and characterization of the carboxylate terminated PAMAM conjugates, their cytotoxicity, and their pharmacokinetics.

The systems were highly water soluble, released platinum slowly *in vitro*, and were 3 to 15 times less toxic than cisplatin. In some cases, however, the conjugate displayed antitumor activity whereas cisplatin was inactive. *In vivo* studies on mice indicated that the accumulation of the conjugate in solid tumor tissue was 50 times higher in comparison to cisplatin. The authors suggested the formation of three main platinated species. Since the release of chlorides in cisplatin can offer up to two bonds to carboxylates, monodentate and bidentate platinum binding structures are possible. Furthermore, an intermolecular bidentate construction causes cross-linking of dendrimers (Fig. 12). Unfortunately, the structural properties of this mixture were not sufficiently defined and characterized.

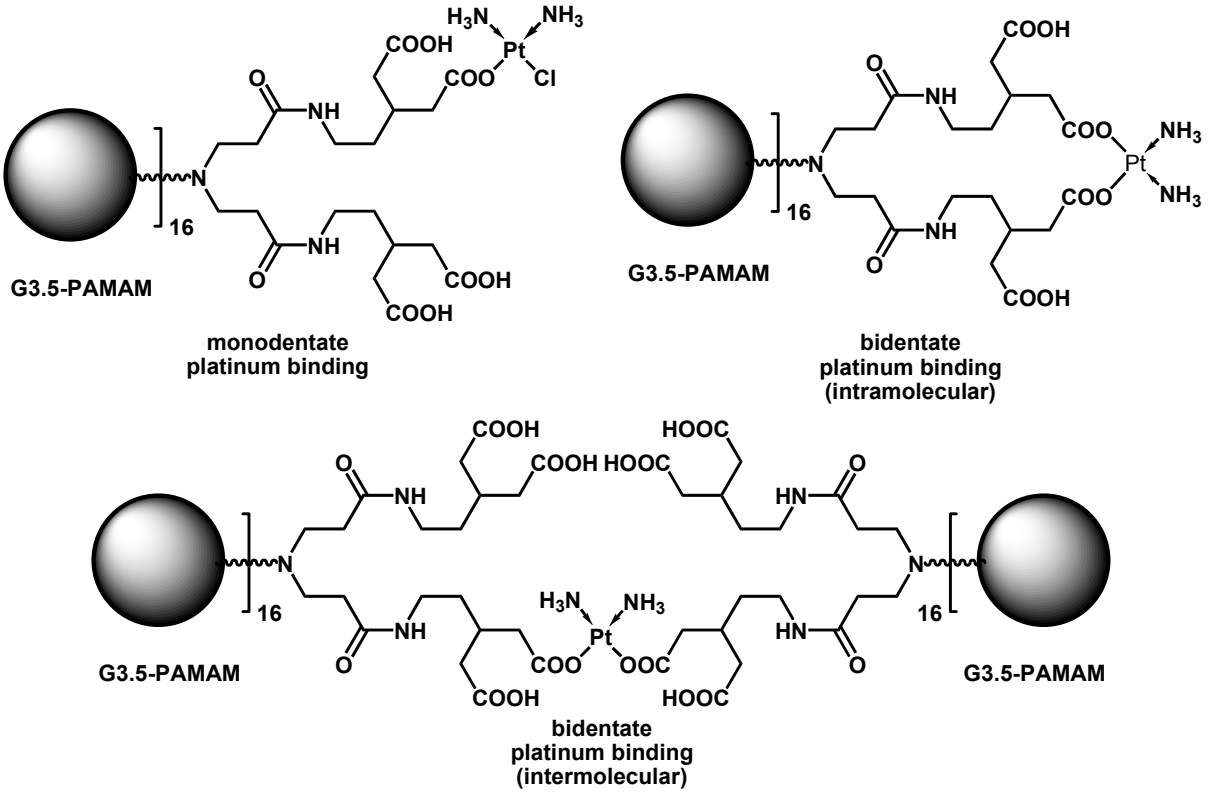


Fig. 12. Structural aspects of PAMAM-platinate.

Baker and coworkers developed nanodevices for drug delivery based on PAMAM dendrimers of generation 5.[156] Their materials were covalently connected to folic acid, FITC, and methotrexate, to study targeting capability, cellular uptake and the efficiency of the drug. These conjugates improved the cytotoxic response of cells to methotrexate 100-fold over the free drug. Recently, Fréchet and coworkers published the most promising dendritic conjugates.[54, 55] Their nontoxic pegylated poly esters were connected to methotrexate using an acid labile hydrazone linkage. It was observed that drug release was a function of pH with an increasing rate at pH < 6.

Another advantage of a carrier, based on a polyester scaffold, is its biodegradability by hydrolytic enzymes. Very recently, Schlüter and coworkers reported the synthesis of a new set of first- and second-generation dendrimers with potential application as drug delivery devices.[157] The periphery of the dendrimers was equipped with different bidentate ligands and a fluorescence tag. Furthermore, well-defined mixed dendrimers, carrying even amounts of the fluorescence tag and a bidentate ligand were synthesized by employing *tris*-orthogonal branching units (Fig. 13). Structure-toxicity relations were determined *in vitro* in concentration-dependent essays using a human breast cancer cell line. The fluorescence tag allowed for monitoring of cellular uptake and intracellular distribution by confocal fluorescence microscopy.

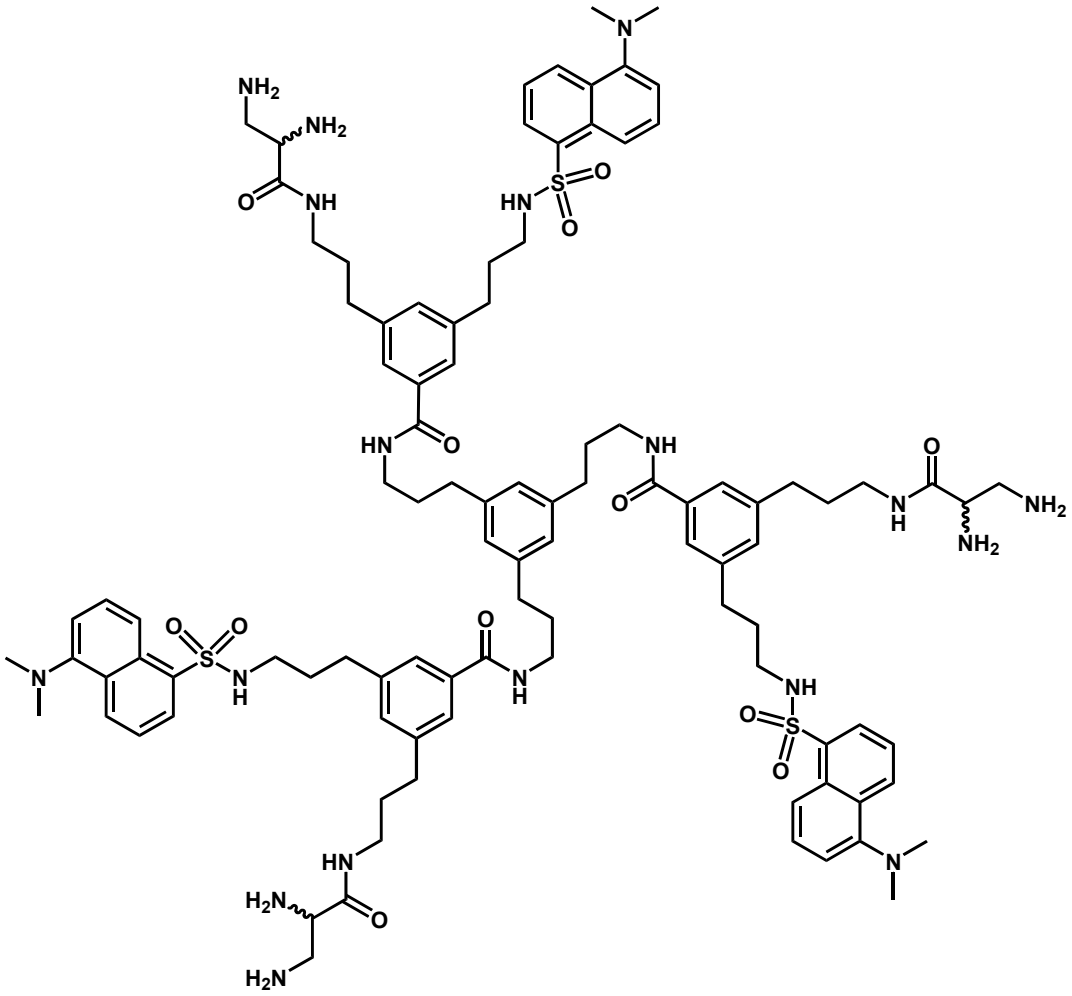
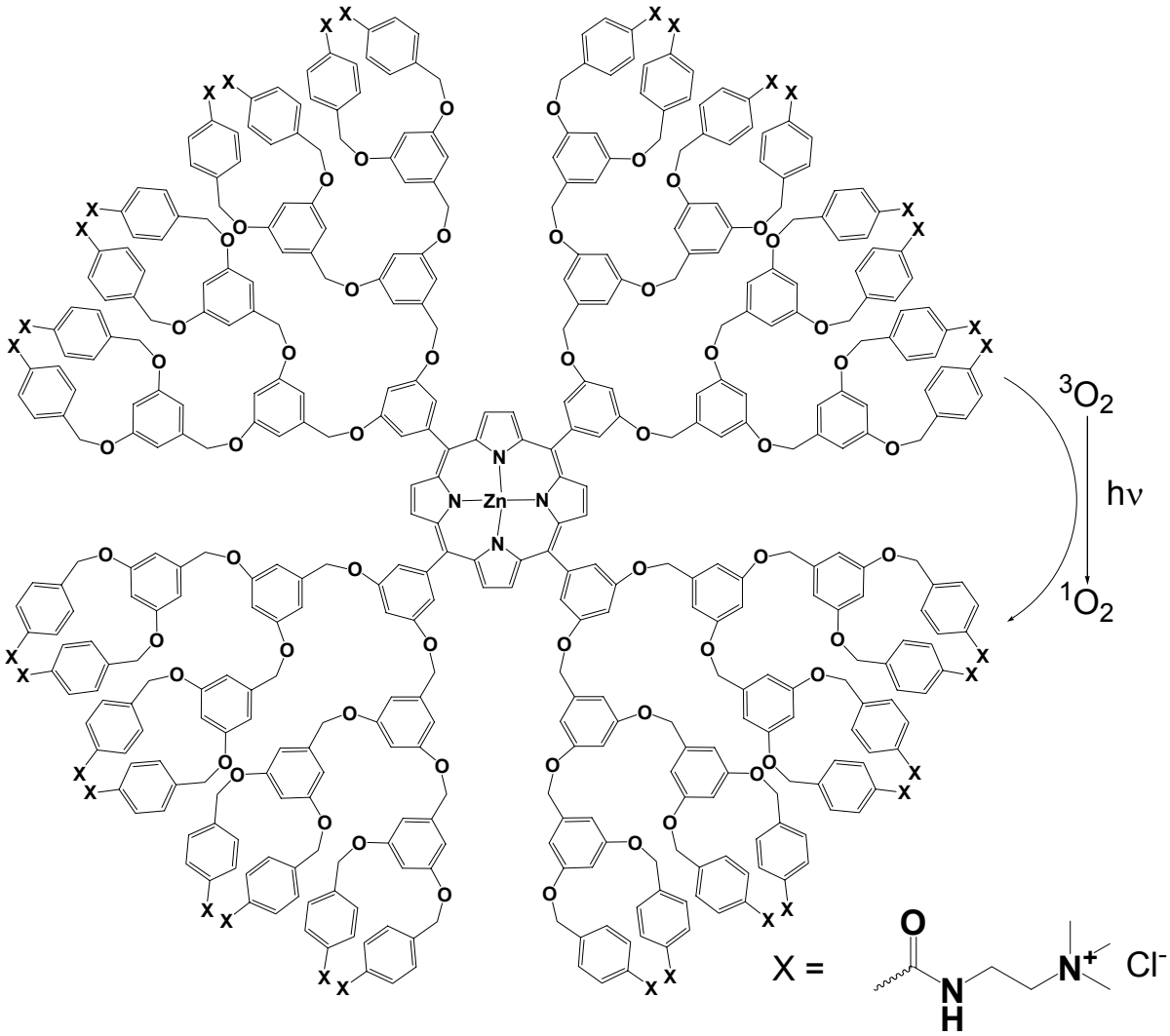


Fig. 13. Dendritic carrier with bidentate ligand and fluorescence tag.

Another aspect of dendritic drug delivery is the photo dynamic treatment (PDT), in which the drug, a photosensitizer, becomes toxic upon irradiation. The *in situ* formation of small amounts of singlet oxygen damages critical elements of neoplastic cells.[158, 159] However, the drug should be non-toxic under non-irradiative conditions.

Edwards and coworkers attached a well-known photosensitizer to the surface of a dendrimer.[160]



Scheme 7. Porphyrin-dendrimer for PDT.

Aida and coworkers published a different approach towards PDT.[161] They used the light-harvesting properties of a poly aryl ether dendrimer with a porphyrin core moiety (Scheme 7). The singlet oxygen-induced cytotoxicity of the dendrimer was increased upon irradiation and 140-fold lower in the dark in comparison to Protoporphyrin IX.