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Review article

Dendrimers as versatile platform in drug delivery applications

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ABSTRACT

About forty percent of newly developed drugs are rejected by the pharmaceutical industry and will never benefit a patient because of poor bioavailability due to low water solubility and/or cell membrane permeability. New delivery technologies could help to overcome this challenge. Nanostructures with uniform and well-defined particle size and shape are of eminent interest in biomedical applications because of their ability to cross cell membranes and to reduce the risk of premature clearance from the body. The high level of control over the dendritic architecture (size, branching density, surface functionality) makes dendrimers ideal carriers in these applications. Many commercial small molecule drugs with anticancer, anti-inflammatory, and antimicrobial activity have been successfully associated with dendrimers such as poly(amidoamine) (PAMAM), poly(propylene imine) (PPI or DAB) and poly(etherhydroxylamine) (PEHAM) dendrimers, either via physical interactions or through chemical bonding ('prodrug approach'). Targeted delivery is possible via targeting ligands conjugated to the dendrimer surface or via the enhanced permeability and retention (EPR) effect. The biocompatibility of dendrimers follows patterns known from other small particles. Cationic surfaces show cytotoxicity; however, derivatization with fatty acid or PEG chains, reducing the overall charge density and minimizing contact between cell surfaces and dendrimers, can reduce toxic effects.

1. Introduction

Every year approximately $65 billion in drug revenues is accounted for by active pharmaceutical ingredients (APIs) with suboptimal bioavailability. The oral drug delivery market with $35 billion is the largest industry segment and is expected to grow as much as ten percent per year. The pulmonary drug delivery market reached $25 billion in 2006 with expected high steady growth in the next five years, and the implantable/injectable delivery market is expected to grow from about $5 billion to over $12 billion by 2010. About forty percent of newly developed APIs are rejected by the pharmaceutical industry and will never benefit a patient because of poor bioavailability due to low water solubility and/or cell membrane permeability. In addition, about seventeen percent of launched APIs exhibit suboptimal performance for the same reasons. Giving the growing impact and need for drug delivery, a thorough understanding of delivery technologies that enhance the bioavailability of APIs is of high importance.

The development of molecular nanostructures with well-defined particle size and shape is of eminent interest in biomedical applications such as delivery of active pharmaceuticals, imaging, or gene transfection. For example, constructs utilized as carriers in drug delivery generally should be in the nanometer range and uniform in size to enhance their ability to cross cell membranes and to reduce the risk of undesired clearance from the body through liver or spleen. Two traditional routes to produce particles that will meet some of these requirements have been widely investigated. The first route takes advantage of the ability of amphiphilic molecules (i.e., molecules consisting of a hydrophilic and hydrophobic moiety) to self-assemble in water above a system-specific critical micelle concentration (CMC) to form micelles. Size and shape of these micelles depend on the geometry of the constituent monomers, intermolecular interactions, and conditions of the bulk solution (i.e., concentration, ionic strength, pH, and temperature). Spherical micelles are monodisperse in size; however, they are dynamic in nature with monomer exchange rates in millisecond to microsecond time ranges. Micelles have the ability to encapsulate and carry lipophilic actives within their hydrocarbon cores. Depending on the specific system, some micelles either spontaneously rearrange to form liposomes after a minor change of solution conditions, or when exposed to external energy input such as agitation, sonication, or extrusion through a filter membrane. Liposomes consist of bilayer lipid membranes (BLMs) enclosing an aqueous core, which can be utilized to carry hydrophilic actives. Furthermore, liposomes with multilamellar membranes provide cargo space for lipophilic actives as well. However, most liposomes are considered energetically metastable and eventually will rearrange to form planar bilayers [1–4]. The second route relies on

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engineering well-defined particles through processing protocols. Examples for this approach include (i) shearing or homogenization of oil-in-water (o/w) emulsions or w/o/w double emulsions to produce stable and monodisperse droplets, (ii) extrusion of polymer strands or viscous gels through nozzles of defined size to manufacture stable and monodisperse micro and nanospheres, (iii) layer-by-layer (LbL) deposition of polyelectrolytes and other polymeric molecules around colloidal cores, resulting in the formation of monodisperse nanocapsules after removal of the templating core, (iv) controlled precipitation from a solution into an anti-solvent, including supercritical fluids, and (v) increasing the surface area per (drug) particle by reducing the particle size. The two last approaches often subject APIs to some form of aggressive processings such as milling, mixing, extrusion, or organic solvent exchange which can reduce the performance of the APIs, especially in case of biomolecules. In addition, there is a limit to particle size reduction from a practical point of view during the production of large drug quantities. Drug particles in the nanometer size, for example, are extremely difficult to stabilize and isolate. Size, degree of monodispersity, and stability of these structures depend on the systems that are being used in these applications [5].

Dendritic polymers or dendrimers provide an alternative route to create very well-defined nanostructures suitable for drug solubilization applications. Dendrimers are core-shell nanostructures with precise architecture and low polydispersity, which are synthesized in a layer-by-layer fashion (expressed in 'generations') around a core unit, resulting in high level of control over size, branching points and surface functionality (Fig. 1). The ability to tailor dendrimer properties to therapeutic needs makes them ideal carriers for small molecule drugs and biomolecules. The three main properties of dendrimers are (i) nanoscale container properties (i.e., encapsulation of a drug), (ii) nano-scaffolding properties (i.e., surface adsorption or attachment of a drug), and (iii) biocompatibility [6–12]. Dendrimers are being considered as additives in several routes of administration, including intravenous, oral, transdermal, and ocular [13]. The biocompatibility of this relatively new class of carrier molecules will be discussed, as well as the use of dendrimers to carry APIs by physical association or chemical bonding (prodrug approach), organized by APIs name.

2. Biocompatibility of dendrimers

A major concern when introducing a new class of nanoparticles for medical applications is directed towards the biocompatibility of these particles. In order to be usable in drug delivery applications, dendrimers have to be non-toxic and non-immunogenic. Most of these studies are very recent, and therefore, the cytotoxicity of dendrimers has been primarily evaluated *in vitro*; however, a few *in vivo* studies have been published [14–18]. As observed for other cationic macromolecules including liposomes and micelles, dendrimers with positively charged surface groups are prone to destabilize cell membranes and cause cell lysis. For example, *in vitro* cytotoxicity, IC_{50} measurements (i.e., the concentration where 50% of cell lysis is observed) for poly(amidoamine) PAMAM dendrimers with amino surface revealed significant cytotoxicity on human intestinal adenocarcinoma, Caco-2 cells [19,20]. Furthermore, the cytotoxicity was found to be generation dependent, with higher generation dendrimers being the most toxic [19,21]. A similar generation dependency of amino-terminated PAMAM dendrimers was observed for the haemolytic effect, studied on a solution of blood cells [22]. However, some recent studies have shown that amino-terminated PAMAM dendrimers exhibit lower toxicity than more flexible linear polymers carrying amine groups, perhaps due to lower adherence of the rigid globular dendrimers to cellular surfaces. The degree of substitution as well as the type of amine functionality is important, with primary amines being more toxic than secondary or tertiary amines [21]. Amino-terminated poly(propylene imine)-PPI dendrimers behave similarly as PAMAM dendrimers with regard to cytotoxicity and haemolytic effects, including the generation-dependent increase both effects [22,23].

Comparative toxicity studies on anionic (carboxylate-terminated) and cationic (amino-terminated) PAMAM dendrimers using Caco-2 cells have shown significantly lower cytotoxicity of the anionic compounds [19]. In fact, lower-generation PAMAM dendrimers possessing carboxylate surface groups show neither haematotoxicity nor cytotoxicity at concentrations up to 2 mg/ml [22]. The biocompatibility of dendrimers is not solely determined by the surface groups. Dendrimers containing an aromatic polyether core and anionic carboxylate surface groups have shown haemolytic activity in a solution of rat blood cells after 24 h. It is suggested that the aromatic interior of the dendrimer may cause haemolysis through hydrophobic membrane contact [22]. However, the influence of the dendrimer core will diminish with increasing dendrimer size (number of generations) and rigidity of the dendritic branches that form the shell around the core. Rigid shells encapsulate the core unit and reduce or prevent interaction between dendrimer core and cell surface.

Partial surface derivatization of cationic dendrimers using chemically inert entities such as PEG or fatty acids is one option to reduce cytotoxicity, as demonstrated on Caco-2 cells. This observation can be explained by the reduced overall positive charge of these surface modified dendrimers. Partial derivatization with as

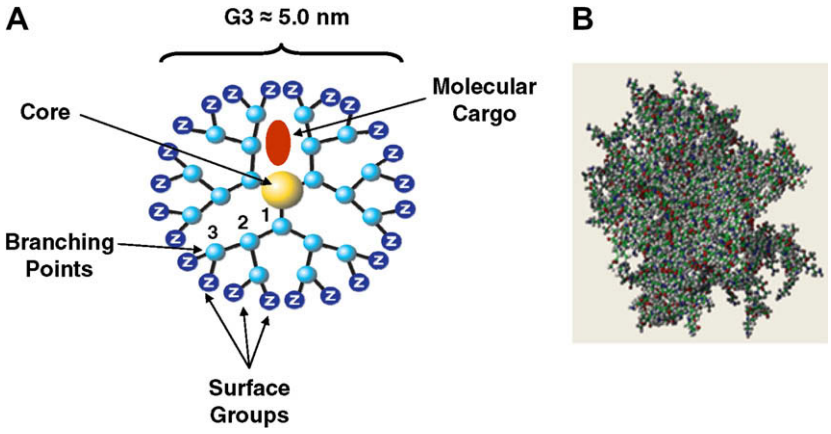

Fig. 1. (A) Schematic 2D presentation of a dendrimer G3 containing three generations (branching points) as indicated by numbers. (B) 3D presentation of dendrimer G3 showing space-filling structure. © 2008 Drug Delivery Solutions LLC.

few as six lipid chains or four PEG chains on G4 PAMAM dendrimers, respectively, was sufficient to substantially lower their cytotoxicity [20]. In some cases, the cytotoxicity of PAMAM dendrimers could be reduced by additives such as fetal calf serum [24].

Only a few systematic studies on the *in vivo* toxicity of dendrimers have been reported so far. Doses of 10 mg/kg of PAMAM dendrimers (up to G5), displaying either unmodified or modified amino-terminated surfaces, injected into mice did not appear to be toxic [25,26]. Polyester-based dendrimers with hydroxyl or methoxy surface have shown low toxicity both *in vitro* and *in vivo*. At very high concentrations (40 mg/ml), these polyester dendrimers induced some inhibition of cell growth *in vitro*, but no increase in cell death was observed. Upon injection into mice, no acute or long-term toxicity problems were observed [27]. Initial immunogenicity studies performed on unmodified G3-7 PAMAM dendrimers with amino surface showed no or weak immunogenicity. However, later studies indicated some immunogenicity of these dendrimers, which could be reduced by surface-modification utilizing PEG chains [28].

Overall, dendrimers show promising biocompatibility. The cytotoxicity of dendrimers with cationic amino surface is similar to that found for liposomes, which have found medical applications (e.g., Doxil®, the liposomal formulation of doxorubicin). Simple surface modifications reducing the overall positive charge greatly enhance the biocompatibility of these dendrimers.

3. Physical association and encapsulation of drugs

APIs can physically interact with dendrimers through either encapsulation into void spaces (nanoscale container) or association with surface groups *Z* (nano-scaffolding) or a mixture of both. Driving forces for these interactions are hydrogen bonding, van der Waals interactions, and electrostatic attraction between opposite charges on dendrimers and APIs [29]. Small organic molecule drugs often are encapsulated into the dendrimers' interior void space, while larger (bio)molecules preferably adsorb onto the dendrimer surface. The following alphabetical listing of drugs physically associated with dendrimers provides an overview of the breadth of the dendritic platform to serve as drug carriers. Research activities are centered on three main classes of drugs: anticancer, anti-inflammatory, and antimicrobial drugs.

3.1. Artemether

Artemether (ART; Fig. 2) is used to treat multi-drug resistant strains of malaria. ART is a lipid-soluble drug developed for oral, rectal and intramuscular use that has been formulated with dendritic micelles to enhance its water solubility. These micelles consisted of hydrophobic di-fluorene methoxycarbonyl-L-lysine (di-FMOC-L-lysine) as the core, conjugated to hydrophilic methoxy poly(ethylene glycol) chains, $MPEG_{2000}$ and $MPEG_{5000}$, forming G0.5 dendrimers. Subsequent generations (G1.5 and 2.5) were formed by using lysine branching units conjugated to the core, followed by capping with MPEG chains. Solubility enhancement between factors 3-fold to 15-fold has been observed, depending on concentration and size of the dendritic micelles. In addition, these formulations increased the stability of ART from 3–5 h to 1–2 days during *in vitro* testing [30].

3.2. Camptothecin

Camptothecin (CPT; Fig. 2) is an anticancer drug that damages DNA, leading to cell destruction. Its therapeutic efficacy is limited by very low water solubility and adverse side effects such as inflammation of the urinary bladder. In attempts to overcome these limitations, camptothecin has been conjugated to polymers such as poly(ethylene glycol) (PEG; prodrug approach), and the drug has been encapsulated into liposomes, micelles, emulsions, and micro- and nanoparticles [31]. Recently, a biocompatible polyester dendrimer composed of natural metabolites, glycerol and succinic acid, has been utilized to encapsulate the camptothecin derivative 10-hydroxy-camptothecin. The cytotoxicity of these dendrimer–drug complexes towards four different human cancer cell lines, human breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (HT-29), non-small cell lung carcinoma (NCI-H460), and glioblastoma (SF-268), has been studied and low IC_{50} (nmol/L) values have been measured. Measurements in MCF-7 cells showed 16-fold increase for cellular uptake and an increase in drug retention within the cell [32]. Dendrimers based on 1,3,5-triazine have also been utilized for the encapsulation of 10-hydroxy-camptothecin. The triazine building block is attractive because of the low cost of the core reagent, 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride), and the ease of displacement of the three chlorine atoms by different amines to generate mono-, di-, and tri-substituted 1,3,5-triazines. Nucleophilic substitution of chlorine atoms with primary or secondary amines in the presence of a base can be controlled with temperature and proceeds as a one-pot procedure. The first chlorine substitution occurs in minutes at 0 °C, the second substitution requires reaction for 12–24 h at ambient temperature, while the third substitution requires the same amount of time but temperatures above 60 °C. These characteristics allow for

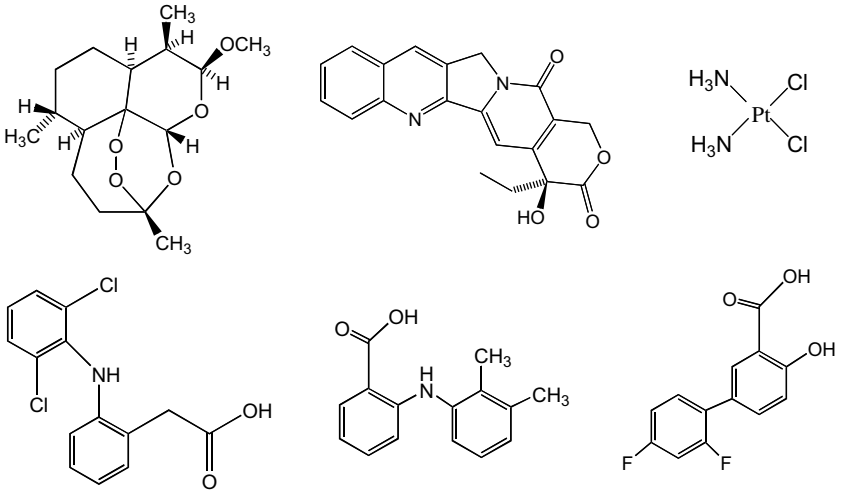

Fig. 2. Structures of drugs (up) artemether, camptothecin, cisplatin; (low) diclofenac, mefenamic acid, and diflunisal.

the preparation of triazine dendrimers with significant structural diversity [33]. Contrary to most dendrimers, triazine dendrimers G2 have been scaled to kilogram quantities, an important requirement for their use in pharmaceutical applications [34]. Mixing of 10-hydroxy-camptothecin with triazine dendrimers resulted in significant solubility enhancement, with 3.7 molecules of drug solubilized per dendrimer. A newly developed dendrimer platform, consisting of poly(etherhydroxylamine) (PEHAM) dendrimers, has been employed to enhance the water solubility of camptothecin [35]. An excess of solid drug was added to a 1% (w/v) solution of PEHAM dendrimer G1, containing 25% (w/w) of PEG_{550} chains conjugated to its surface, in water or aqueous alcohol (20% v/v) and stirred for several hours. Excess solid drug was removed by filtration, while dissolved but free drug was removed by brief dialysis. The amount of camptothecin associated with the dendrimers was measured by UV absorbance. The solubility of camptothecin was enhanced 19-fold in water and 27-fold in aqueous alcohol. Lyophilized PEHAM–CPT formulations quickly dissolved in phosphate buffer solution (pH 7.2) or saline solution, depending on dendrimer size and surface functionality (Fig. 3).

3.3. Cisplatin

The anticancer drug cisplatin (Fig. 2) exerts its effects by forming stable DNA–cisplatin complexes through intrastrand cross-links, resulting in alteration of the DNA structure that prevents replication and initiates apoptosis. The therapeutic effect of cisplatin is limited by its poor water solubility (1 mg/ml), low lipophilicity, and the development of drug resistance. Encapsulation of cisplatin within PAMAM dendrimers resulted in complexes with slower release, higher accumulation in solid tumors, and lower toxicity than free cisplatin. Preliminary studies gave cisplatin loadings of 15–25 wt% for PAMAM dendrimers generation 3.5 (size ~3.5 nm; M_W ~13 kDa). The tumor activity of Cisplatin–dendrimer complexes was studied using B16F10 cells, subcutaneously injected into C57 mice to provide a solid tumor model. After approx. 12 days, when tumors had grown to a mean area of 50–100 mm^2, the animals were injected with a single dose of either cisplatin or cisplatin–dendrimer complex (1 mg/kg cisplatin for both formulations). At certain time points within 48 h, animals were culled and blood and tissue samples were taken. Compared to cisplatin alone, cisplatin–dendrimer complexes were found to accumulate preferentially at the tumor site. The tumor area under the curve (AUC) for these complexes was 5-times higher than that for free cisplatin, while accumulation in the kidney only increased 2.4-times, and accumulation in the liver was reduced (Table 1) [36,37].

3.4. Diclofenac and mefenamic acid

Diclofenac and structurally closely related mefenamic acid (Fig. 2) are taken to reduce inflammation and act as analgesic. Both non-steroidal anti-inflammatory drugs (NSAIDs) have been encap-

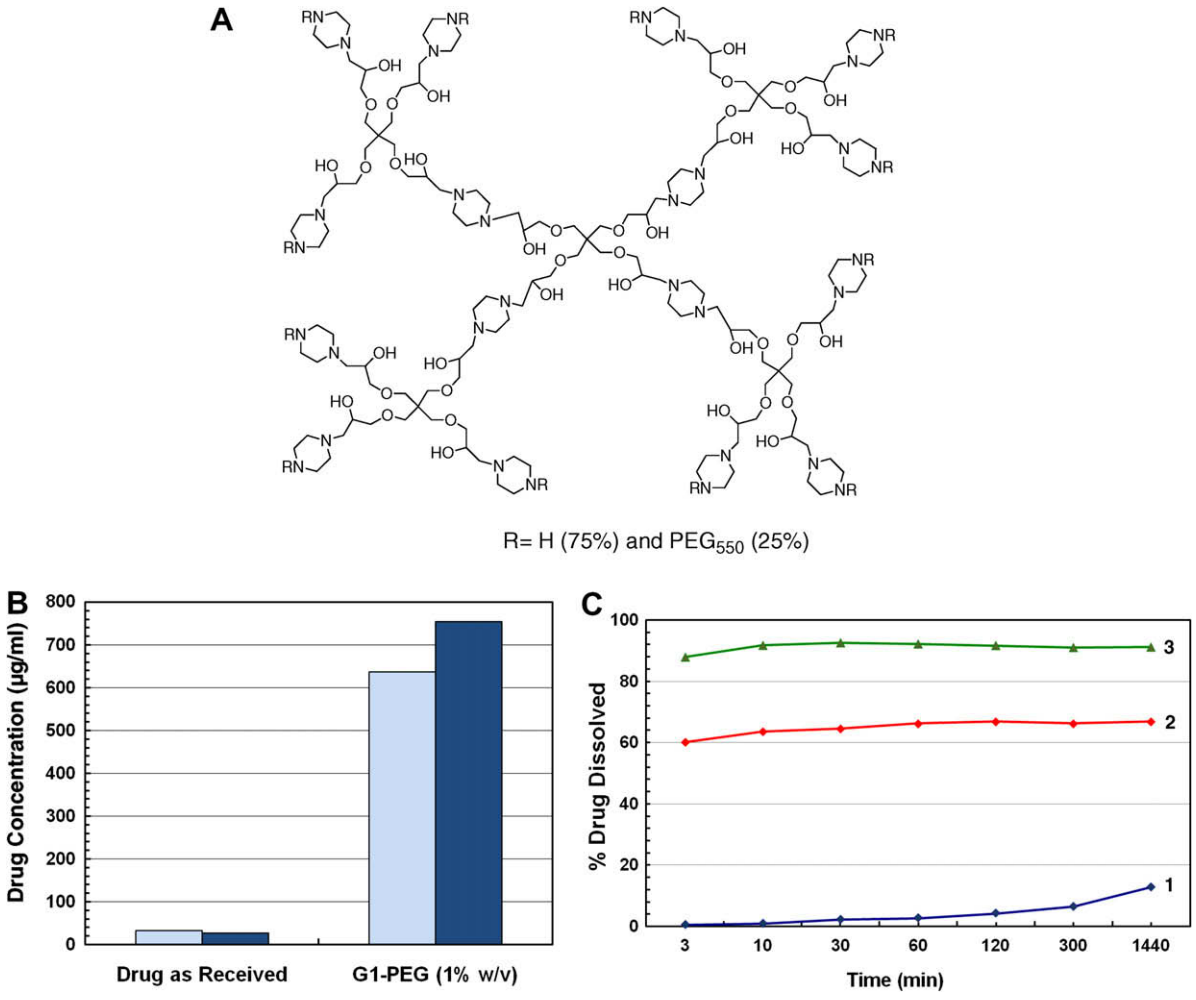

Fig. 3. (A) Structure of a poly(etherhydroxylamine) (PEHAM) dendrimer G1 conjugated with 25% of PEG_{550} to its surface. (B) Solubility enhancement of camptothecin formulated with PEHAM dendrimer G1-PEG_{550} in water (light) and water–alcohol (dark) compared to drug as received. (C) Dissolution curves of free drug (1) and lyophilized drug formulations with PEHAM dendrimers G1-PEG_{550} (2) and G3-OH (3). © 2007 Dendritic Nanotechnologies, Inc.

Table 1
AUC value (μg Pt/ml blood or μg Pt/organ) over 48 h; 5 mice/data point.

Organ	Cisplatin	Cisplatin–dendrimer complex
Tumor	5.3	25.4
Blood	9.4	10.7
Liver	51.6	17.0
Kidney	57.6	138.1

sulated into dendrimers built from citric acid-poly(ethylene glycol)-citric acid triblock copolymers. Citric acid, a tricarboxylic acid carrying a hydroxyl group at its middle carbon atom, has been used to build dendrons generations 1–3 through ester formation. Two dendrons were then attached to PEG_{600} dicarboxylic acid as the core unit. Drug encapsulation was performed in tetrahydrofuran, a decent solvent for the drugs, by stirring at room temperature, followed by precipitation of the complexes from hexanes and diethyl ether. Between 15 and 33 mol% of diclofenac and 27 to 36 mol% of mefenamic acid have been encapsulated into dendrimers G1-3 [38].

3.5. Diflunisal

Diflunisal (Fig. 2) is a generic NSAID with similar therapeutic profile compared to diclofenac and mefenamic acids. Its adverse side effects during oral administration, such as local or systemic disturbance in the gastrointestinal tract, have limited clinical applications of this drug. PAMAM dendrimers have been selected to facilitate transdermal delivery of diflunisal. *In vitro* permeation studies with excised rat skins indicated significantly enhanced permeation of diflunisal, resulting in 2.48-fold higher blood level concentrations for the diflunisal–PAMAM complex than for the pure drug suspension [39]. In a similar study, PAMAM dendrimers G2-4 have been used to investigate the potential of these structures to increase the solubility of diflunisal and related NSAIDs, ketoprofen, ibuprofen, and naproxen. The extremely low water solubility of these NSAIDs has been significantly improved by PAMAM dendrimers. For example, after mixing with PAMAM dendrimer G4 at a concentration of 10 mg/ml, diflunisal solubility increased 26-fold, from 0.19 to 4.94 mg/ml. The apparent solubility of these API increased linearly as a function of PAMAM concentration over the range 0–5 mg/ml. At higher concentrations, the solubility increase was slightly lower than predicted because of precipitation of insoluble drug–PAMAM complexes. The observed order of increased solubility in PAMAM dendrimers at a constant dendrimer concentration and generation was naproxen > ketoprofen > ibuprofen > diflunisal [40].

3.6. Dimethoxycurcumin

Curcumin and dimethoxycurcumin (Fig. 4) are principal curcuminoids of the Indian curry spice turmeric. Their anticancer effects stem from the ability to induce apoptosis in cancer cells without cytotoxic effects on healthy cells. Curcumins can interfere with the activity of the transcription factor NF-κB, which has been linked to a number of cancer diseases. For improved water solubility, dimethoxycurcumin has been mixed with PAMAM dendrimers generations 3.5 and 4.0. The maximum drug incorporation efficiencies were 4.3 and 5.0 M for PAMAM G3.5 and G4.0, respectively. FTIR-ATR studies have indicated that dimethoxycurcumin adopted its enolic form within the dendrimers [41].

3.7. Doxorubicin

Doxorubicin (DOX; Fig. 4) is a DNA-intercalating anthracycline drug widely used in chemotherapy to treat some leukemia, Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, and multiple myeloma. Doxorubicin has been successfully encapsulated into liposomes (trade name Doxil®) to enhance drug solubility and reduce side effects. However, liposome formulations have potential shelf-life stability problems, and therefore, doxorubicin has been encapsulated into PAMAM dendrimers G3 and G4, which had PEG-monomethyl ether chains of molecular weights 550 and 2000 Da conjugated to their surfaces via urethane bonds. The encapsulation efficiency was dependent on PEG chain length and size of the dendrimer, with the highest encapsulation efficiency of 6.5 doxorubicin molecules per dendrimer found for PAMAM G4-PEG_{2000}. Drug release from this dendrimer was sustained at low ionic strength, again reflecting PEG chain length and dendrimer size, but fast in isotonic solution [42]. To further modulate and control release and activity, doxorubicin has been associated with PAMAM G4 dendrimers, followed by encapsulation of this complex into liposomes made from hexadecylphosphocholine, egg yolk phosphatidylcholine, and stearyl-

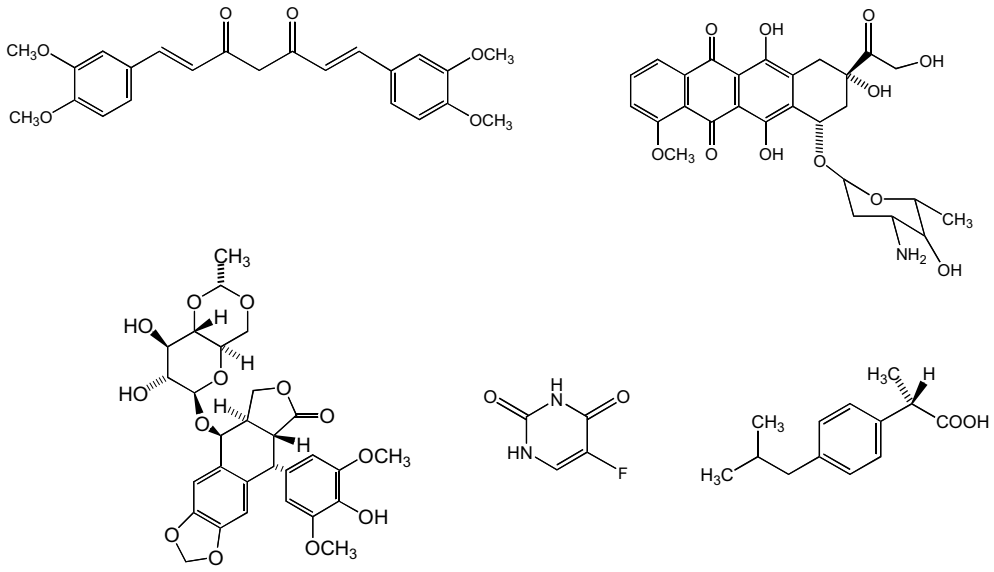

Fig. 4. Structures of drugs (up) dimethoxycurcumin and doxorubicin; (low) etoposide, 5-fluorouracil, and ibuprofen.

amine in 10:10:0.1 molar ratio. DOX loading into PAMAM G4 (3:1 molar ratio) was about 97%, while encapsulation efficiency of the DOX–PAMAM complex into liposomes was 91%. The release of doxorubicin from dendrimer and liposome–dendrimer formulations was slow; however, the liposome–dendrimer formulation showed high activity against eight cancer cell lines (DMS114 and NCI-H460 – small cell lung cancer and non-small cell lung cancer; HT29 and HCT116 – colon; MDA-MB435 and MCF-7 – breast; SF268 – central nervous system; DU145 – prostate; and SF268 – central nervous system). The DOX–PAMAM formulation without liposomes was active at first but quickly lost activity, while a DOX–PAMAM formulation with liposomes consisting just of egg yolk phosphatidylcholine and stearylamine in 10:0.1 molar ratio was mostly inactive [43]. Micelles composed of a 10 kDa molecular weight poly(ethylene oxide) (PEO) block attached to a polyester dendrimer G3 with cyclic acetals of 2,4,6-trimethoxybenzaldehyde conjugated to its surface have been studied as DOX delivery vehicle. Hydrolysis of the cyclic acetals at acidic pH such as those encountered in tumor tissue and in endosomes and lysosomes released 2,4,6-trimethoxybenzaldehyde and revealed polar 1,3-diol moieties on the dendrimer periphery. This change in solubility of the dendrimer block triggered the disruption of the micelle and release of DOX. *In vitro* toxicity studies revealed that empty micelles were relatively non-toxic, while DOX encapsulated in micelles showed toxicity quite similar to that of the free drug. However, laser scanning confocal microscopy images indicated the localization of DOX in intracellular organelles in contrast to free DOX, which is localized in the cell nucleus after 24 h, suggesting that DOX-loaded micelles are indeed taken up by cells, but that the mechanism of action of the released drug may differ from that of the free drug [44].

3.8. Etoposide

Etoposide (Fig. 4) is an inhibitor of the enzyme topoisomerase II. It is used for the treatment of malignancies such as Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiform. It is often given in combination with other drugs, and therefore, its low solubility poses a formulation challenge. In order to increase the water solubility of etoposide, micelles composed of block copolymers, lipophilic poly(ε-caprolactone) (PCL) and hydrophilic PEG_{5000}, conjugated to a generation two PAMAM-OH dendrimer as the core, have been synthesized. The chemical bonding between dendrimer core and copolymer chains was chosen to enhance the stability of the micellar drug carrier. Micelle disintegration upon dilution in the blood stream is a major set-back for this form of drug delivery. The loading capacity of these micelles achieved with etoposide was up to 22% (w/w). A cytotoxicity assay (porcine kidney epithelial cells, LLC-PK) suggested that PCL-PEG-PAMAM micelles alone were not toxic in cell culture, whereas etoposide incorporated into micelles displayed significant cytotoxic activity, comparable with that of the free etoposide [45]. Two other drugs have been encapsulated into these micelles as well, doxorubicin and the anti-inflammatory drug indomethacin.

3.9. 5-Fluorouracil

5-Fluorouracil (5-FU; Fig. 4) is a pyrimidine analogue that belongs to the family of drugs called antimetabolites. Some of its principal use is in colorectal and pancreatic cancers, in which it has been the established form of chemotherapy for decades. As a pyrimidine analogue, 5-FU is transformed inside the cell into different cytotoxic metabolites, which are then incorporated into DNA and RNA, inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. A conjugate between PAMAM G4 dendrimer and PEG_{5000} chains has been utilized in order to improve the solubility of fluorouracil. Drug loading into this PEGylated PAMAM was enhanced 12-fold compared to the respective non-PEGylated dendrimer, while release rates were enhanced 6-fold, allowing sustained release of 5-fluorouracil over a period of six days from PEGylated PAMAM G4 [46].

3.10. Ibuprofen

Ibuprofen (IPA, Fig. 4) is a NSAIDs that inhibits both COX-1 and COX-2. It appears that its analgesic and anti-inflammatory activities are achieved through COX-2 inhibition, whereas COX-1 inhibition is responsible for unwanted side effects on platelet aggregation and gastrointestinal mucosa. However, at low doses, ibuprofen appears to have the lowest incidence of gastrointestinal adverse reactions of all the non-selective NSAIDs. As mentioned earlier, the solubility of ibuprofen can be significantly enhanced by encapsulation into PAMAM dendrimers (e.g., PAMAM G4 at a concentration of 10 mg/ml, IPA solubility increased 74-fold from 0.10 to 7.45 mg/ml) [40]. In another study it was found that association of IPA with PAMAM dendrimers G3 and G4 resulted in association of 78 ibuprofen molecules per PAMAM dendrimer through electrostatic interactions between dendrimer surface amine groups and the carboxyl group of the drug. In contrast, only up to 24 drug molecules were encapsulated into a hyperbranched polyester having approximately 128 hydroxyl surface groups [47]. Ibuprofen was successfully transported into A549 human lung epithelial carcinoma cells by PAMAM dendrimers. Dendrimers with either amino or hydroxyl surface groups entered the cells faster (approx. 1 h) than the pure drug. The anti-inflammatory effect of IPA–PAMAM complexes was demonstrated by more rapid suppression of COX-2 mRNA levels than that achieved by the pure drug [48].

3.11. Indomethacin

Indomethacin (Fig. 5) is a member of the arylalkanoic acid class of NSAIDs, which includes diclofenac. Indomethacin is poorly soluble in water and sparingly soluble in alcohol. Formulating indomethacin with PAMAM dendrimers G4 with amino, hydroxyl, and carboxylate surface groups enhanced the water solubility of the drug (~25 μg/ml) by factors 29-fold (amine), 26-fold (hydroxyl) and 10-fold (carboxylate) [49,50]. The high efficiency of amino-surface dendrimers can be explained by the presence of the carboxylic acid function in indomethacin, allowing for strong electrostatic and hydrogen bond interactions between drug and carrier. Anionic PAMAM dendrimers G3.5 with carboxylate surface groups have been conjugated with PEG_{3350} chains at different degrees of substitution. The PEG chains carried folic acid at one end to provide the ability for targeted delivery to activated macrophages. Folate-PEG conjugation increased the loading efficiency of indomethacin by factors 10- to 20-fold, depending on the degree of substitution. Tissue distribution studies in arthritic rats revealed significantly lower indomethacin uptake by the stomach for the folate-PEG conjugates compared to free drug, thereby limiting gastric-related side effects [51]. Poly(etherhydroxylamine) (PEHAM) dendrimers are even more efficient solubility enhancers for indomethacin. At low concentrations (0.3–0.5% w/v) in water, PEHAM dendrimers G1 with hydroxyl, piperazine (secondary amine) and partially PEGylated surfaces (for structure see Fig. 3) enhanced the water solubility of indomethacin by factors 48-fold (hydroxyl), 88-fold (amine), and 136-fold (PEG_{550}). These PEHAM dendrimers, therefore, have shown higher efficiencies at lower-generation than PAMAM dendrimers (G1 versus G3.5 and G4), resulting in significantly reduced cost for dendrimer-based formulations. It is noteworthy that the dependency between concentration of dissolved drug and dendrimer concentration is linear for PEGylated PEHAM, while in case

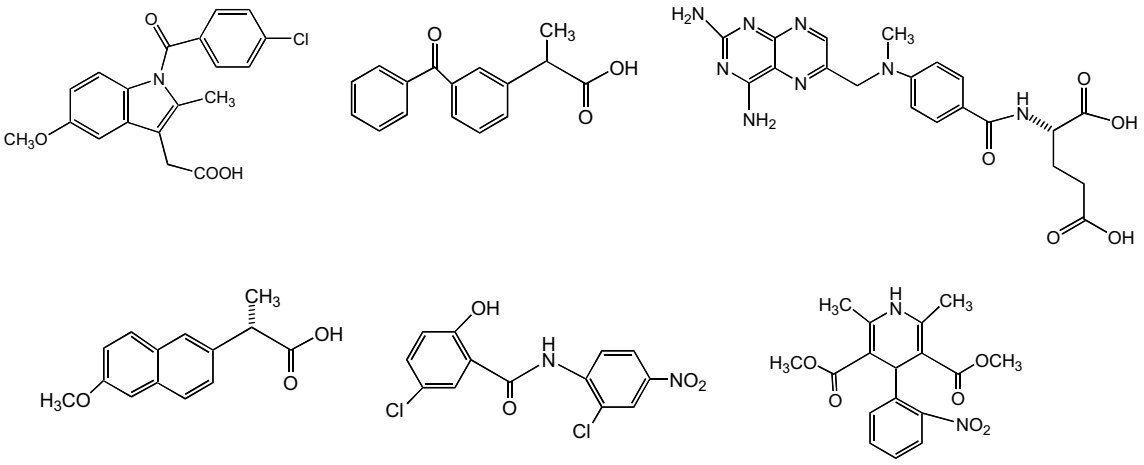

Fig. 5. Structures of drugs (up) indomethacin, ketoprofen, and methotrexate; (low) naproxen, niclosamide, and nifedipine.

of PEHAM dendrimers with hydroxyl and amine surfaces the dependency is approaching a plateau. This different behavior can be explained by cluster formation at higher concentrations in case of dendrimers with surfaces able to form hydrogen bonds, reducing the available total surface area for drug uptake. However, cluster formation is prevented in the presence of PEG chains, resulting in the linear solubility enhancement found for indomethacin.

3.12. Ketoprofen

Ketoprofen (Fig. 5) belongs to the propionic acid class of NSAIDs with analgesic and antipyretic effects. The presence of PAMAM dendrimers enhanced the transdermal delivery of ketoprofen, leading to 2.73-fold higher bioavailability compared to suspensions of the pure drug [39]. The solubility of ketoprofen was significantly enhanced by association with PAMAM dendrimers (e.g., PAMAM G4 at a concentration of 10 mg/ml, ketoprofen solubility increased 19-fold from 0.88 up to 16.92 mg/ml) [40]. Under fixed pH condition, the solubility of ketoprofen in PAMAM G4 solutions increased in an approximately linear manner with the dendrimer concentration. The same behavior was found for PAMAM dendrimers G2, G3, and G5, with the solubility of ketoprofen increasing with the size of the dendrimers. For all PAMAM dendrimer solutions, the solubility of ketoprofen was highest at pH 6 and lowest at pH 3. Therefore it was assumed that the solubility enhancement was due to electrostatic interactions between PAMAM surface amine groups and the carboxyl group of ketoprofen [52]. *In vivo* studies in mice showed a prolonged pharmacodynamic behavior for ketoprofen–PAMAM complexes, suggesting PAMAM dendrimers as potential drug carrier of ketoprofen with sustained release behavior under suitable conditions [53].

3.13. Methotrexate

Methotrexate (MTX; Fig. 5), an antimetabolite and antifolate drug used in the treatment of many cancers, acts by inhibiting the metabolism of folic acid. Methotrexate is a weak dicarboxylic acid, and therefore, mostly negatively charged at physiologic pH. Its mean oral bioavailability spans the range of 13–76%, while the mean intramuscular bioavailability is 76%. The higher doses of MTX often used in cancer chemotherapy can cause toxic effects to the rapidly dividing cells of bone marrow and gastrointestinal mucosa. Similar to doxorubicin, methotrexate has been encapsulated into generations 3 and 4 PAMAM dendrimers, which had PEG_{550} and PEG_{2000} monomethyl ether chains conjugated to their surfaces to modify bioavailability and toxicity. The encapsulation efficiency was dependent on PEG chain length and size of the dendrimer, with the highest encapsulation efficiency of 26 MTX molecules per dendrimer found for PAMAM G4-PEG_{2000}. This efficiency for MTX was 4-fold higher than that of DOX, reflecting the stronger interaction between negatively charged MTX molecules and ammonium-containing core of the dendrimer; however, drug release from this dendrimer had the same challenge as observed for DOX, i.e., sustained release at low ionic strength but fast release in isotonic solution [42]. In a more recent study employing PAMAM dendrimer G3 with different amounts of PEG_{2000} conjugated to its surface, it was reported that the percentage of surface coverage with PEG_{2000} chains had little influence on the encapsulation efficiency of methotrexate (between 13.2 and 16.4 molecules per dendrimer), but resulted in reduced release rates (between 75 and 450 h) [54]. The effects of alterations in the chemical structure of polyester-*co*-polyether (PEPE) dendrimers on encapsulation and release of methotrexate were investigated and MTX loading as high as 24.5% (w/w) was found, depending on the number of branches and the presence of aromatic rings as branching units. Reducing the number of branching units and aromatic rings drastically lowered the loading with MTX. Physical entrapment, weak hydrogen bonding, and hydrophobic interactions were established to be the mechanisms of encapsulation. Release of MTX was biphasic, including burst release over six hours followed by sustained release over a period of 50 to 168 hours. Initial burst and release rate could be reduced by increasing the number of branching units and aromatic rings [55]. Approximately three molecules of MTX were encapsulated into 1,3,5-triazene dendrimers G3, carrying 48 amine groups on their surfaces, by physical mixing of drug and dendrimer in saline solution and sonication of the mixtures until clear solutions had formed (Fig. 6). MTX and MTX–dendrimer solutions (2 mg/kg MTX) were administered to male C3H mice via intraperitoneal (i.p.) injection for three consecutive days. Forty-eight hours after dosing, the mice were sacrificed and serum was collected for biochemical analyses. The levels of alanine transaminase (ALT) were used to probe liver damage. When the drugs were encapsulated by the dendrimer, a significant reduction in hepatotoxicity was observed; ALT levels in mice administered with MTX–dendrimer solution were 27% lower than those of animals treated with the drug alone [56]. Physical association and chemical conjugation of methotrexate hve been compared using PAMAM G5 dendrimers. Supporting previous studies, it was found that the dendrimer–MTX inclusion complex showed minimal release of free MTX (less than 5%) when dialyzed against water; however, when dialyzed against phosphate buffered saline (PBS), more than 70% of MTX was released within 2.5 h. This trend was consistent and reproducible

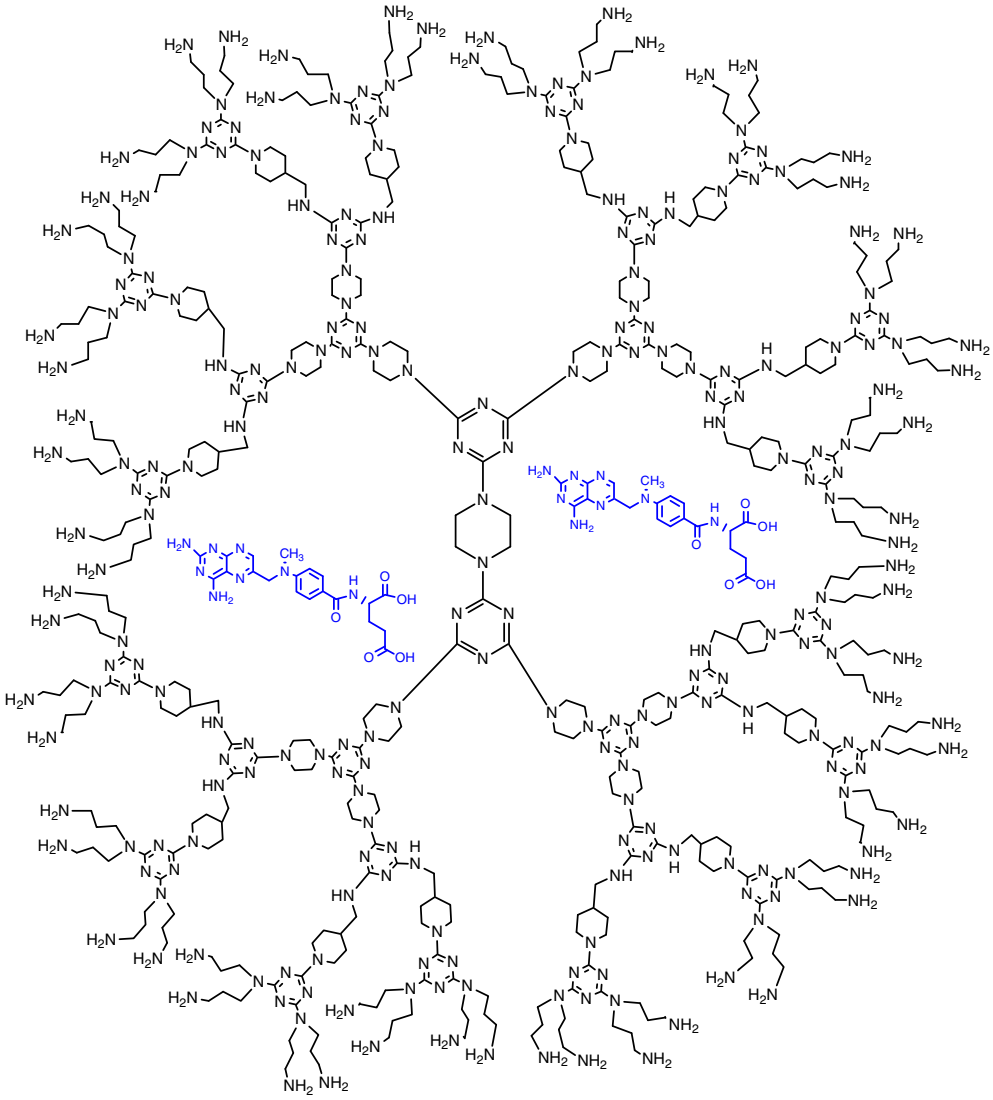

Fig. 6. Structure of a 1,3,5-triazene dendrimer encapsulating two molecules of methotrexate. Core size and bond lengths are not to scale to accommodate the 2D presentation of a 3D molecule. Adapted from [56].

with hydroxyl, acetyl, or amine surface PAMAM G5 dendrimers, although the rate of release was slightly different, indicating that the interactions between dendrimer and MTX were not strong enough to retain MTX inside the complexes when the physical binding forces were neutralized in buffered salt solution. In contrast, MTX chemically bonded to dendrimers was not released in water or PBS [57]. These observations clearly indicate that the mode of dendrimer–drug interaction has to be selected based on the desired application: if the objective is enhanced water solubility for formulation purposes, then physical association between drug and dendrimer (i.e., dendrimer serving as an excipient) would be the method of choice; however, if the objective is drug delivery, which requires that drug and dendrimer remain together for some time, then chemical bonding between drug and dendrimer (i.e., dendrimer serving as a carrier) would be required.

3.14. Naproxen

The NSAID naproxen and its sodium salt (Fig. 5) are commonly used for the reduction of moderate to severe pain, fever, inflammation and stiffness caused by conditions such as osteoarthritis, rheumatoid arthritis and others. There are very few studies involving naproxen and dendrimers, almost all using the prodrug approach. However, the solubility of naproxen was significantly enhanced by the association with PAMAM dendrimers (e.g., PAMAM G4 at a concentration of 10 mg/ml, naproxen solubility increased 1570-fold from 0.02 to 31.41 mg/ml) [40].

3.15. Niclosamide

The antimicrobial drug niclosamide (Fig. 5) is a member of the anthelmintic family primarily utilized to treat tapeworm infections. Niclosamide is practically insoluble in water at physiological pH and becomes sparingly soluble over the range of pH 8–10. The drug is currently provided as a suspension formulation based on neat niclosamide or its salts. Mixing of niclosamide with PAMAM dendrimers containing a carboxylate surface had no effect on the water solubility; however, mixing with PAMAM dendrimers (8.0 mM) containing a primary amine surface significantly enhanced the water solubility of niclosamide by factors 372-fold (G0), 1354-fold (G1), 1945-fold (G2) and 6176-fold (G3). In all but the last case the drug–PAMAM ratio was 1:1, while for G3 the calculated ratio was 4:1. The observed solubility increase was related to electrostatic interactions between positive charged

nitrogen atoms within the PAMAM dendrimers and negatively charged drug molecules. Consequently, the solubility enhancement changed with the pH of the bulk solution. The enhancement factors using dendrimers were considerably higher than the factor observed for hydroxypropyl-β-cyclodextrin (~10-fold enhancement). Dissolution studies revealed that rate and extent of dissolution of niclosamide were significantly improved from mixtures with PAMAM dendrimers and cyclodextrin; however, the rate of dissolution was slower from the dendrimer compared to cyclodextrin mixtures, indicating strong interactions between the drug and PAMAM-amine dendrimers [58].

3.16. Nifedipine

The NSAID nifedipine (Fig. 5) is a dihydropyridine calcium channel blocker. The effect of dendrimer size and surface group functionality on the aqueous solubility of nifedipine has been studied using PAMAM dendrimers G0–3 with amino surface and G0.5–2.5 with ester surface at different pH. Solubility enhancement of nifedipine was higher in the presence of ester-terminated dendrimers than that their amino-terminated analogues possessing the same number of surface groups. Not unexpected, the solubility of nifedipine increased with the size of the dendrimers. For example, at pH 7.0 the sequence of G2.5 > G3 > G1.5 > G2 ⩾ G0.5 > G1 > G0 was reported [59]. In a related study, PAMAM–nifedipine complexes have been embedded into hydroxypropylmethyl cellulose (HPMC) gels. Nifedipine release from these gels was measured and compared to formulations containing the common cosolvent isopropanol. PAMAM dendrimers enhanced the release rate of nifedipine, dependent on dendrimer size and concentration. The increase in solubility and release rate was lower than observed for isopropanol formulations; however, the presence of PAMAM dendrimers prevented the recrystallization of nifedipine observed in formulations containing isopropanol [60]. The combination between hydrogel and dendritic building blocks has been extended by using dendritic junctions based on poly(benzyl ether) dendrons in hydrogels formed by PEG segments (PEG_{3400}). Encapsulation and release profiles of these constructs have been evaluated using dye molecules such as bromophenol blue, *m*-cresol purple, crystal violet, and methylene blue as model compounds. The weight swelling ratios of these amphiphilic hydrogels were sensitive to changes in the pH of the surrounding medium, with the highest values observed under acidic conditions. Gel binding and release capabilities were dependent on the size of the dendritic junctions, the pH of the surrounding medium, and the nature of the substrate. The highest binding was achieved with systems containing G3 dendritic junctions, while the fastest release of the same compounds was observed for hydrogels with G0 junctions [61]. Solubility enhancement experiments using API molecules have not been reported thus far; however, these dendrimer–hydrogel hybrids seem to offer promising options for drug delivery applications.

3.17. Paclitaxel

The anticancer drug paclitaxel (PTX; Fig. 7) is a mitotic inhibitor used in chemotherapy to treat patients with lung, ovarian, breast, and head and neck cancers as well as advanced forms of Kaposi's sarcoma. The drug works by interfering with normal microtubule growth during cell division, which especially affects fast growing cancer cells. In order to enhance its poor water solubility, paclitaxel has been encapsulated mainly into micelle-based formulations [62–65]. There is one recent study employing dendrimers as solubility enhancer. PTX encapsulation into polyglycerol dendrimers resulted in 400-fold improved water solubility compared to the pure drug [66]. However, most dendrimer-related formulations utilize PTX conjugation to the dendritic carrier (prodrug approach).

3.18. Quinolones nadifloxacin and prulifloxacin

Quinolones (Fig. 7) are a family of broad-spectrum antibiotics covering a host of aerobic Gram-negative, Gram-positive and even some anaerobic species responsible for various infections (e.g., prostatitis, tuberculosis, pneumonia, bronchitis, urinary tract, and respiratory tract). The majority of quinolones in clinical use belong to the subset of fluoroquinolones, carrying a fluoro atom attached to the central ring system. The bactericidal quinolones are divided into generations based on their antibacterial spectrum. Nadifloxacin belongs to the second-generation quinolones, while prulifloxacin is a prodrug in development (Phase III) that converts in the body to the active form ulifloxacin following oral administration. Ulifloxacin, a compound that inhibits bacterial DNA replication, has demonstrated activity against a wide range of bacteria that can cause infectious diarrhea. Limited water solubility of quinolones prevents the design of liquid dosage forms and restricts their use in topical applications. The solubility of nadifloxacin and prulifloxacin was significantly enhanced in formulations containing PAMAM dendrimers G3–5 with amine surface, increasing in an approximately linear manner with dendrimer concentrations. Driving forces for drug–PAMAM interactions were electrostatic attraction between PAMAM-amine surface groups and carboxylate groups in the drug molecules, hydrophobic interactions between dendrimer cavities and drugs, and hydrogen bonds between tertiary amines within the PAMAM core and drug molecules. Drug–PAMAM complexes still display antibacterial activity against *Escherichia coli*. Interestingly, when equal amounts of free prulifloxacin and prulifloxacin complexed to G4 PAMAM dendrimer were tested, it was found that prulifloxacine–PAMAM complexes were more potent by a factor 2-fold than the free drug in DMSO. The enhanced antibacterial activity could not be contributed to the dendrimer itself as pure PAMAM G4 is known to displayed antibacterial activity against *E. coli* at a much higher concentration [67].

3.19. Silver salts

The encapsulation of silver salts within PAMAM dendrimers produced conjugates exhibiting slow silver release rates and antimicrobial activity against various Gram-positive bacteria [68]. PAMAM dendrimers G4 with ethylenediamine (EDA) core and tris(2-hydroxymethyl)amidomethane (TRIS) surface and G5, EDA core with carboxylate surface, were used. Silver-containing PAMAM complexes were prepared by adding aqueous solutions of the dendrimers to the calculated amount of silver acetate powder. Although silver acetate is hardly soluble in water, it quickly dissolved in these PAMAM solutions. This enhancement is due to the combined action of silver carboxylate salt formation and/or complex formation between silver ions and internal dendrimer nitrogens. This procedure resulted in slightly yellow dendrimer-complex/salt solutions, that very slowly photolyzed when exposed to light into dark brown, metallic silver-containing dendrimer–silver nanocomposite solutions. Final sample concentrations were confirmed by atomic absorption spectroscopy. For antimicrobial testing, the standard agar overlay method was used. In this test, dendrimer–silver compounds were examined for diffusible antimicrobial activity by placing a 10-μL sample of each solution onto a 6-mm filter paper disk and applying the disk to a dilute population of the test organisms, *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. The silver–dendrimer complexes displayed antimicrobial activity comparable or better than those of silver nitrate solutions. Interestingly, increased antimicrobial activity was observed with dendrimer carboxylate salts, which was attributed to the very high local concentration (256 carboxylate groups around a 5.4 nm diameter sphere) of nanoscopic size silver composite particles that are accessible for microorganisms. The

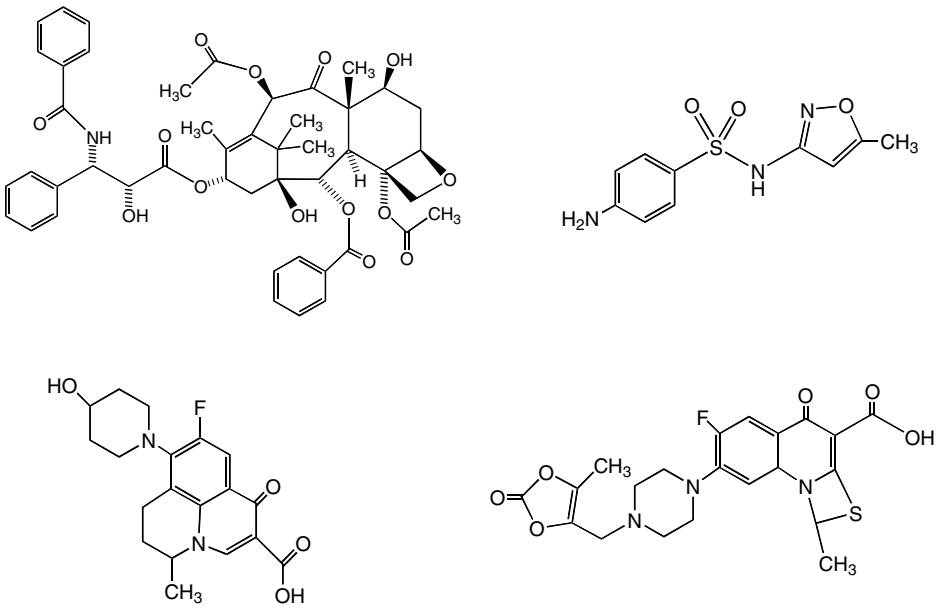

Fig. 7. Structures of drugs (up) paclitaxel and sulfamethoxazole; (low) quinolones nadifloxacin and prulifloxacin.

antimicrobial activity was lower when internal silver complexes were applied instead of silver adducts to the surface, indicating that the accessibility of the silver is an important factor.

3.20. Sulfamethoxazole

Sulfamethoxazole (SMZ; Fig. 7) is a sulfonamide antibacterial drug with primary activity against susceptible forms of *Streptococcus*, *Staphylococcus aureus*, *Escherichia coli*, *Haemophilus influenza*, and oral anaerobes. SMZ is sparingly soluble in water, causing problems in its clinical applications. Association with PAMAM dendrimers G2-4 in low (3–9 mM) concentration range enhanced the aqueous solubility of sulfamethoxazole linearly with the dendrimer concentration (e.g., PAMAM G3 at a concentration of 10 mg/ml, SMZ solubility increased by a factor 40-fold compared to double-distilled water at 37 °C). Microbiology studies showed that SMZ–PAMAM complexes increased the antibacterial activity by factors 4-fold and 8-fold compared to pure SMZ dissolved in dimethyl sulfoxide and sodium hydroxide solutions. These observations indicate that PAMAM dendrimers might be considered as potential drug carriers of sulfonamide-based antibiotics [69].

3.21. Poly(ethylene glycol) (PEG) Shells

In many of the studies described above, conjugation of PEG shells around dendrimers has improved the physical association between drug molecules and dendritic carriers. To study the effect of PEG chain length, pyrene was incorporated into G3 PAMAM dendrimer carrying 32 primary amine groups on its surface, which were conjugated to PEG_{750}, PEG_{2000}, and PEG_{5000} chains. Pyrene is a valuable molecular probe for fluorescence spectroscopy because of its high quantum yield and lifetime, and the sensitivity of its fluorescence emission spectrum to the polarity of its environment. Proton NMR analysis revealed slightly decreasing numbers of PEG chains covering the dendrimer surface (28 PEG_{750} *versus* 25 PEG_{2000} *versus* 23 PEG_{5000}), which has been explained by increasing steric hindrance. Solubility enhancement of pyrene in water was insignificant for G3 PAMAM dendrimer without PEG chains. The presence of a PEG_{750} shell enhanced pyrene solubility by a factor ~10-fold (from 10^{-7} to 10^{-6} M), while the PEG_{2000} derivative increased pyrene solubility by a factor 40-fold as measured by fluorescence spectroscopy. The presence of a PEG_{5000} shell resulted in reduced pyrene solubility compared to PEG_{2000}, presumably because the longer chains shielded the dendrimer from pyrene uptake [70].

4. Chemical conjugation of drugs to dendrimers (prodrug approach)

Physical interactions between dendrimers and APIs have specific features; (1) they leave the APIs unaltered, and therefore, provide a less challenging regulatory path forward; (2) they are easy to establish but provide limited control over release kinetics; and (3) they often allow only limited drug loading, resulting in rather poor drug-to-dendrimer ratios. Alternatively, drugs can be conjugated to dendrimers via chemical bonding [71]. There are three main pathways to create these prodrugs: (1) direct conjugation of drugs to the dendrimer surface; (2) conjugation via a linker molecule if the drugs do not carry the desired functional group for direct conjugation or if the linker molecules are needed to modify solubility profiles or release kinetics, or reduce congestion of drug molecules on the dendrimer surface, allowing a higher degree of conjugation; and (3) drug molecules can become an integral part of the dendritic carrier that is released through certain triggering events at the desired location, e.g., a tumor site. Added benefits of the prodrug approach consist in modified pharmacokinetics and pharmacodynamics. The pharmacodynamic behavior of two amide-bond linked PAMAM–methotrexate (Fig. 5) prodrugs was evaluated, one prodrug prepared by coupling carboxyl-terminated PAMAM G2.5 dendrimer via the 2-amine group at the aromatic ring of MTX (prodrug A), the other prodrug synthesized by coupling amino-terminated PAMAM G3 dendrimer via the γ-carboxyl group of MTX (prodrug B). Prodrug A with MTX payload of 2.8 exhibited significant anticancer activity towards MTX-resistant cell lines, while prodrug B with much higher MTX payload of 22.4 showed no cytotoxicity on these cells. This behavior was explained by the different ionic nature of these prodrugs, resulting in different residence times within the lysosomes, and therefore, different intracellular drug release profiles [72].

4.1. Enhanced oral absorption through p-gp efflux pump inhibition

Another benefit of PAMAM dendrimer prodrugs consists of enhanced intestinal drug absorption by inhibiting P-glycoprotein (P-gp) efflux pump activity. Recent observations on intestinal absorption of drugs suggested that an intestinal secretion process mediated by P-gp limits the bioavailability of drugs that are P-gp substrates. Dendrimers have the ability to cross cell barriers by both paracellular and transcellular pathways. Therefore propranolol, a poorly water-soluble drug that is a substrate for the P-gp efflux transporter, was conjugated to PAMAM dendrimer G3 in an attempt to enhance the transport of propranolol across Caco-2 cells. This PAMAM–propranolol prodrug indeed was shown to bypass the efflux system [73,74]. In a related study, prodrugs between the smaller and less toxic PAMAM dendrimer G1 and the water-insoluble P-gp substrate terfenadine (Ter) were synthesized using succinic acid (suc) or succinyl-diethylene glycol (suc-deg) as a linker (Fig. 8). All of the PAMAM–terfenadine prodrugs were more hydrophilic than the parent drug. The influence of the dendrimer prodrugs on the integrity and viability of Caco-2 cells was determined by measuring the transepithelial electrical resistance (TEER) and leakage of lactate dehydrogenase (LDH) enzyme, respectively. The LDH assay indicated that the dendrimer prodrugs had no impact on the viability of Caco-2 cells up to a concentration of 1 mM; however, the IC_{50} of the prodrugs was lower than that of PAMAM dendrimer G1 because of the high toxicity of terfenadine. Transport of dendrimer prodrugs across monolayers of Caco-2 cells showed an increase in the apparent permeability coefficient (P_{app}) of terfenadine in both apical-to-basolateral (A → B) and basolateral-to-apical (B → A) directions, with the apparent permeability coefficient for (A → B) significantly greater than (B → A) (Fig. 8) [73].

4.2. L-DOPA (3,4-dihydroxy-L-phenylalanine) dendrimers

L-DOPA (levodopa, 3,4-dihydroxy-L-phenylalanine) is a prodrug capable of passing the blood–brain barrier and treating Parkinson's disease. It is decarboxylated in the brain to become dopamine, the neurotransmitter, by the enzyme aromatic-L-amino acid decarboxylase. However, it also induces side effects such as dystonia and dyskinesia after large doses or chronic use. Slow release of L-DOPA has shown reduction of the problems associated with its long-term therapy. L-DOPA was therefore converted into well-defined, monodisperse dendritic macromolecules. A third-generation L-DOPA dendrimer contained 30 L-Dopa residues, which made up its core, branches, and periphery. Individual L-Dopa moieties in the dendrimer were connected to one another via hydrolysable diester linkages. These Dopa dendrimers showed a 20-fold increase in aqueous solubility and enhanced photostability in solutions over L-Dopa under identical conditions (Fig. 9). Hydrolysis of these novel dendritic prodrugs was studied by nuclear magnetic resonance (NMR) spectroscopy and high pressure liquid chromatography (HPLC), showing sequential degradation for these dendritic prodrugs [75].

4.3. Doxorubicin prodrugs

Attachment of anticancer drugs to polymers improves their passive targeting to tumors because of the increased permeability of tumor vasculature to macromolecules and the decreased lymphatic drainage from the tumor (enhanced permeation and retention (EPR) effect). Passive targeting decreases the systemic toxicity and enhances the therapeutic efficacy of conjugated drugs. The antitumor effect of doxorubicin (DOX; Fig. 4) conjugated to an asymmetric, biodegradable polyester dendrimer (8–10 wt% DOX) was evaluated in mice bearing C-26 colon carcinomas. The design of the dendritic carrier optimized (1) blood circulation time through size and molecular architecture; (2) drug loading through multiple attachment sites; (3) solubility through attachment of PEG chains; and (4) drug release through the use of pH-sensitive hydrazone linkages. In culture, the dendrimer–DOX prodrug was more than 10 times less toxic than free DOX towards C-26 colon carcinoma cells after exposure for 72 h. Upon i.v. administration

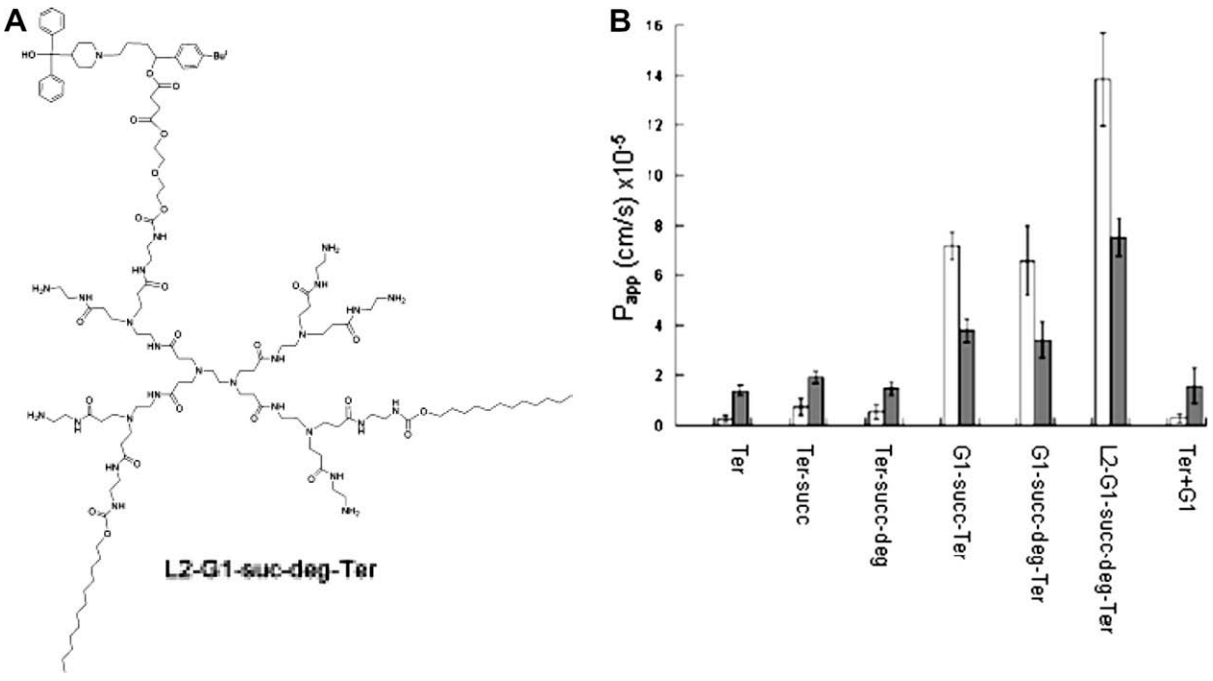

Fig. 8. (A) Structure of PAMAM dendrimer G1 conjugated with the drug terfenadine via a succinic acid linker (G1-suc-Ter). (B) Apical-to-basolateral (A → B) (□) and basolateral-to-apical (B→A) (■) permeability after 3 h across Caco-2 cell monolayers at 37 °C of free terfenadine (Ter), conjugates with succinic acid (succ) and diethylene glycol (deg) linkers and lauroyl (L2) chains, and terfenadine in the presence of G1 PAMAM dendrimer (Ter + G1) (mean ± SD, $n = 4$). Reproduced with permission from [73]. © 2007 American Chemical Society.

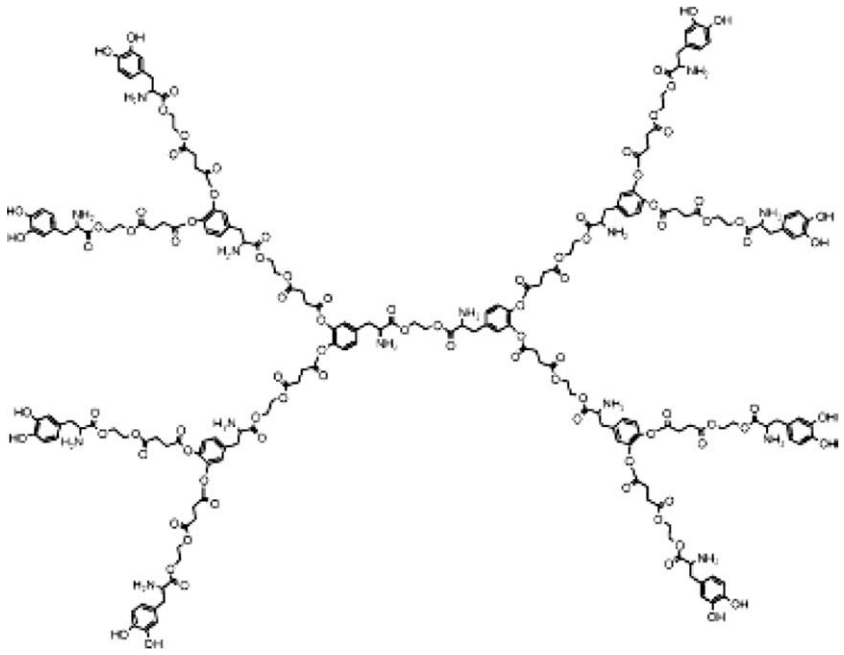

Fig. 9. Structure of second-generation L-DOPA dendrimer prodrug. Reproduced with permission from [75]. © 2006 American Chemical Society.

to BALB/c mice with subcutaneous C-26 tumors, dendrimer–DOX prodrugs were eliminated from the serum with a half-life time of 16 ± 1 h, and tumor uptake was 9-fold higher than i.v. administered free DOX at 48 hours. In efficacy studies performed with BALB/c mice bearing subcutaneous C-26 tumors, a single i.v. injection of dendrimer–DOX prodrugs at 20 mg/kg DOX equivalents 8 days after tumor implantation caused complete tumor regression and 100% survival of the mice over the 60-day experiment. No cures were achieved in tumor-implanted mice treated with free DOX at its maximum tolerated dose (6 mg/kg), drug-free dendrimer, or dendrimer–DOX prodrugs in which DOX was attached via a stable carbamate bond. The antitumor effect of hydrolysable dendrimer–DOX was similar to that of an equimolar dose of liposomal DOX (Doxil®) [76].

4.4. Epirubicin prodrugs

Dendrimers based on amino adipic acid or beta-glutamic acid as branching molecules and built on PEG diols of molecular weight 10,000 Da have been investigated. The large polycyclic drug epirubicin was chosen as a model to investigate the influence of branching and steric hindrance during coupling reactions with the drug. Several derivatives with increased numbers of drug molecules linked to each PEG chain have been synthesized and their physical, chemical and biological properties have been studied (Fig. 10). Most drug-loaded conjugates dissolved in water only after the prodrugs had been pre-dissolved in dimethyl sulfoxide (DMSO). This solubility problem was solved by adding a hydrophilic peptide linker (tetrapeptide GGRR) between the drug and the polymer. These prodrugs showed better stability than free epirubicin in different pH buffers and in plasma, as well as prolonged residence time in blood using mice due to reduced kidney clearance. Dynamic light scattering investigation showed that these prodrugs had a high tendency to aggregate into stable micelles with hydrodynamic radii ranging approximately from 38 to 46 nm [77].

4.5. Methotrexate prodrugs

The effect of amide linker orientation within PAMAM-methotrexate prodrugs has been discussed earlier [72]. In a related study,

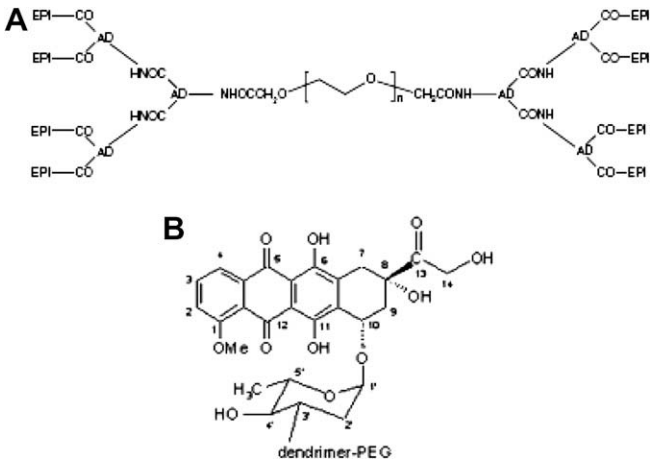

Fig. 10. (A) Structure of PEG dendrimer with amino adipic acid branching units (AD), conjugated to the drug epirubicin (EPI). (B) Structure of epirubicin.

MTX has been conjugated via an ester bond to PAMAM dendrimers G5 with partially acetylated amine surface. Partial surface acetylation was done to reduce the overall cationic character of this surface and prevent non-specific binding of these prodrugs to cell surfaces *in vivo*. Remaining amino groups have been utilized to conjugate folic acid (FA) via an amide linkage as an active targeting ligand and the dye fluorescein isothiocyanate (FITC) via a thiourea linkage as an imaging moiety to the dendrimer surface (Fig. 11). These trifunctional therapeutic nanodevices, G5-FITC-FA-MTX, have been studied *in vitro* using folic acid receptor-expressing KB cancer cells, and a time- and dose-dependent inhibition of cell growth has been observed. Only targeted dendrimer conjugates containing folic acid inhibited cell growth in KB cells, whereas non-targeted prodrugs without folic acid failed to induce growth inhibition. These studies show the potential of targeted prodrugs for growth suppression of tumor cells that overexpress FA receptors [78,79]. In an attempt to verify the *in vitro* results, the G5-FITC-FA-MTX conjugates were injected i.v. into immunodeficient nude mice bearing human KB tumor xenografts that overexpress

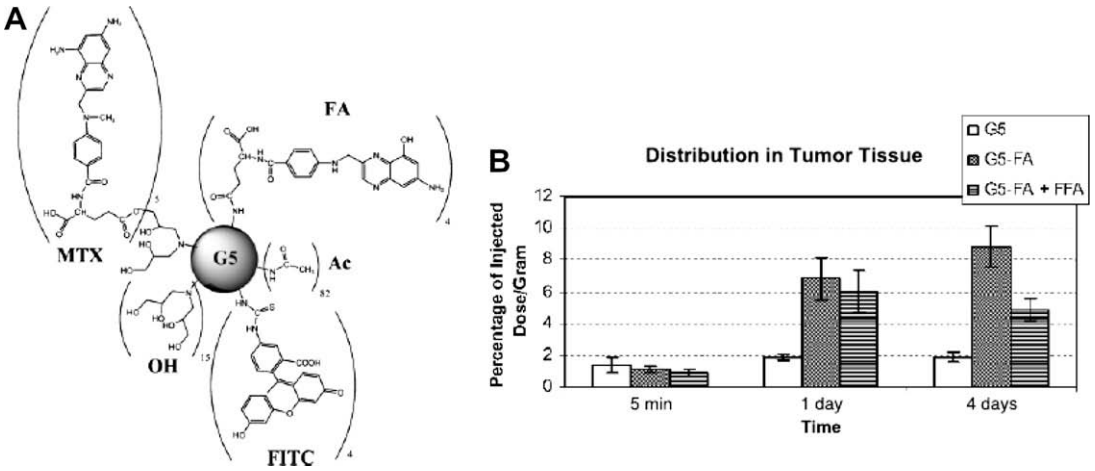

Fig. 11. (A) Schematic representation of PAMAM dendrimer G5 surface-functionalized with FITC, FA, and MTX. (B) Tumor tissue distribution of PAMAM dendrimer G5 without and with folic acid (FA) ligand before and after addition of free folic acid (FFA) to block FA receptors present on some non-tumor cell surfaces. Reproduced with permission from [79,80]. © 2005 American Chemical Society; and © 2005 American Association for Cancer Research.

the folic acid receptor. The blood concentration of tritium-labeled G5-3H-FA decreased from 29.1% ID/g at 5 min to 0.2% ID/g at 1 day. The tissue distribution in several organs, including lung, heart, pancreas, and spleen, showed a trend similar to blood concentration. These organs do not express folate receptors, and therefore, did not show significant differences between the non-targeted and the targeted dendrimers. The concentrations of both prodrugs, G5-3H and G5-3H-FA, in the brain were low at all time points, suggesting that the polymer conjugates did not cross the blood–brain barrier. Although the kidneys are the major clearance organ for these dendrimers, they are also known to express high levels of the folate receptor on their tubules. The level of non-targeted G5-3H in the kidneys decreased rapidly and was maintained at a moderate level over the next several days. In contrast, the level of G5-3H-FA increased slightly over the first day most likely due to folate receptors present on the kidney tubules. This increase was followed by a decrease over the next several days as the compound was cleared through the kidneys. Both prodrugs, G5-3H and G5-3H-FA, were rapidly excreted within 24 h following injection, primarily through the kidneys. Confocal microscopy images showed that labeled dendrimer prodrugs were present within the cytosol of targeted cells. This observation was consistent with the *in vitro* binding and internalization studies of these prodrugs. It also raised the possibility of releasing the drug within the targeted cells due to endosomal disruption of the acid-labile drug linker. Important to note that all mice were observed for the duration of the studies for signs of dehydration, inability to eat or drink, weakness, or change in activity level. No gross toxicity, either acutely or chronically up to 99 days, was observed regardless of whether the dendrimer conjugate contained methotrexate. The weight was monitored throughout the experiment and no loss of weight was observed; in fact, the animals gained weight. At each time point, a gross examination and histopathology of the liver, spleen, kidney, lung, and heart were done. No morphologic abnormalities were observed on the histopathology examination. No *in vivo* toxicity was noted in any animal group following the dendrimer injection [80].

In another study employing PAMAM dendrimer G5, the monoclonal antibody Cetuximab (also known as C225), which targets both the epidermal growth factor receptor (EGFR) and its mutant form EGFRvIII, was covalently linked via its Fc region to the dendrimer. This targeting vector carried also 12.6 molecules of methotrexate per unit of dendrimer as measured by mass spectrometry and UV/vis spectroscopy. The major limitation in using anti-EGFR monoclonal antibodies clinically to treat patients with brain tumors has been that only extraordinarily small quantities reached the tumor following systemic administration. This fact has been attributed to a combination of their rapid clearance by the reticuloendothelial system and the blood–brain barrier, which prevents the passage of hydrophilic agents with a molecular weight more than 250 Da unless there is an active transport system. Specific binding and cytotoxicity of the C225-G5-MTX bioconjugate were evaluated against the EGFR-expressing rat glioma cell line F98(EGFR). Cetuximab alone was not cytotoxic to F98(EGFR) cells at the concentration tested, whereas the IC_{50} of the bioconjugate was 220 nmol/L, which was a 2.7 log unit decrease in toxicity over that of free methotrexate. The biodistribution of C225-G5-MTX in rats bearing brain implants of F98(EGFR) gliomas was determined 24 h following convection-enhanced delivery of ^{125}I-labeled bioconjugate. At this time, 62.9 ± 14.7% ID/g tumor was localized in rats bearing F98(EGFR) gliomas, thereby showing specific molecular targeting of the tumor. However, animals that received C225-G5-MTX, cetuximab, or free methotrexate had median survival times of 15, 17, and 19.5 days, respectively, which were not statistically different from each other or untreated control animals. The outcome of this study clearly demonstrated that specific molecular targeting is but one of several requirements that must be fulfilled if an antibody–drug bioconjugate will be therapeutically useful [81].

4.6. Naproxen prodrugs

The NSAID naproxen (Fig. 5) was conjugated to PAMAM dendrimer G0 as a model for poorly water-soluble drugs to enhance drug solubility and bioavailability for oral delivery applications. Conjugation was carried out either directly via an amide bond or via ester bonds using L-lactic acid or diethylene glycol as linker molecules. All prodrugs were more hydrophilic than naproxen alone. pH-dependent studies revealed that amide and ester linkages were stable in buffer solutions at pH between 1.2 and 8.5. However, naproxen was enzymatically released from both ester conjugates in 80% human plasma. The lactide ester hydrolyzed slowly releasing about 25% of naproxen within 24 h, while the diethylene glycol ester hydrolyzed quickly ($t_{1/2}$ = 51 min). In 50% rat liver homogenate both esters hydrolyzed more quickly, with $t_{1/2}$ = 180 min for lactide ester and $t_{1/2}$ = 4.7 min for diethylene glycol ester. The naproxen prodrugs were non-toxic when exposed to Caco-2 cells. Permeability studies across Caco-2 monolayers at 37 °C showed significant enhancement in the transport of naproxen-dendrimer prodrugs [82,83]. In another approach, naproxen

was conjugated to unsymmetrical poly(arylester) dendrimers to prepare complexes with enhanced water solubility of the drug and access for hydrolytic cleavage of the bond between drug and carrier. Detailed results on the biological evaluation of these complexes have not been reported [84].

4.7. Paclitaxel prodrugs

Paclitaxel (PTX; Fig. 7) has been conjugated through its hydroxyl group in 2′-position to the same PAMAM dendritic carrier as methotrexate, giving G5-FITC-FA-PTX. Concentration and time needed for this dendrimer conjugate to induce cytotoxic effects in KB cells have been determined. KB cells with up- and down-regulated folic acid receptors were treated with the trifunctional dendrimer conjugate or control dendrimer conjugate without PTX at concentrations of 100, 50, and 25 nM for 1 h, then the treatment was washed out and cells were incubated in fresh medium for an additional 72 h before examination under a fluorescent microscope. Dendrimer conjugates at concentrations higher than 100 nM were cytotoxic to both types of KB cells. Only KB cells with up-regulated folate receptor and treated either with the trifunctional dendrimer conjugate or the control dendrimer conjugate showed green fluorescence, documenting uptake of the dendrimer conjugates. Although these *in vitro* results are promising, *in vivo* experiments still need to be conducted [85]. A related study employed PAMAM dendrimer G4 with biocompatible hydroxyl surface for the conjugation of paclitaxel via a succinic acid linker. The results were compared to paclitaxel conjugated to a linear PEG chain as the carrier. Both these polymers increased the aqueous solubility of PTX. The aqueous solubility of paclitaxel is limited to 0.3 μg/mL; however, the solubility of the paclitaxel-bis (PEG) conjugate was observed to be 2.5 mg/mL, while the solubility of the PAMAM-paclitaxel conjugate was further improved to 3.2 mg/mL. Although conjugation of paclitaxel to both traditional PEG polymer and PAMAM G4-SA dendrimer improved the bioavailability of the drug, the influence of the conjugation on anticancer activity of paclitaxel depended on the type of drug carrier. Conjugation to PEG polymer significantly decreased the toxicity of paclitaxel as evaluated using A2780 human ovarian carcinoma cells. In fact, the IC_{50} dose of PEG-PTX conjugate increased more than 25-fold when compared with free drug. The resulting decrease in anticancer activity might be explained by the increase in molecular mass of the whole complex, which in turn changed the mechanism of the cellular internalization of PEG-PTX polymer from diffusion (free drug) to endocytosis. In contrast, conjugation of paclitaxel to PAMAM G4-SA dendrimer substantially enhanced cytotoxicity of the drug, leading to a decrease in the IC_{50} dose by more than 10-fold when compared with free drug. Taken together, the data obtained suggest that PAMAM G4-SA dendrimers represent a promising vehicle for intracellular delivery of paclitaxel. The developed dendrimer prodrugs provide both cytoplasmic and nuclear delivery of therapeutics and enhance anticancer activity of paclitaxel [86]. Sixteen PTX molecules have been conjugated to a 1,3,5-triazene dendrimer G2 via glutaric ester linkages, followed by PEGylation with NHS-$mPEG_{2000}$ and NHS-$mPEG_{5000}$ to give PTX-dendrimer-PEG conjugates of molecular weights 46 kDa (∼14 PEG_{2000} chains) and 77 kDa (∼12 PEG_{5000} chains). The composition of these two drug carriers was 30 wt% PTX, 52 wt% PEG and 18 wt% dendrimer, and 18 wt% PTX, 71 wt% PEG, and 11 wt% dendrimer, respectively. The degree of drug loading for the first PTX-dendrimer-PEG conjugate corresponds to the concentrations achieved in Cremophor EL, the clinically relevant castor oil derivative used for solubilizations [87]. However, *in vitro* or *in vivo* data on these conjugates have not been reported.

4.8. Carborane prodrugs – boron neutron capture therapy (BNCT)

Boron neutron capture therapy is a cancer treatment based on a nuclear capture reaction. When ^{10}B is irradiated with low energy or thermal neutrons, highly energetic α-particles and ^{7}Li ions are produced that are toxic to tumor cells. To achieve the desired effects, it is necessary to deliver ^{10}B to tumor cells at a concentration of at least 10^9 atoms per cell. High levels of boron accumulation in tumor tissue can be achieved by using boronated antibodies that are targeted towards tumor antigens. However, this approach can impair the solubility and targeting efficiency of the antibodies. One study, involving intratumoral injection of a conjugate between PAMAM dendrimer G5 carrying 1100 boron atoms at its surface and cetuximab, the monoclonal antibody specific for the EGF receptor, showed that the conjugate was present at an almost 10-fold higher concentration in brain tumors than in normal brain tissue [88]. To reduce the liver uptake observed for boronated PAMAM dendrimer conjugates, PEG chains were attached onto the dendrimer surface in addition to the borane clusters to provide steric shielding. As compared with a dendrimer without PEG chains, the amount of liver uptake was found to be less for PEG-conjugated dendrimers with an average of 1.0–1.5 chains of PEG_{2000} but higher for dendrimers with 11 chains of PEG_{550}. Folic acid moieties were also conjugated to the ends of the PEG chains to enhance the uptake of the dendrimers by tumors overexpressing folate receptors (Fig. 12). Although this strategy was successful in enhancing localization of the molecules to tumors in mice bearing

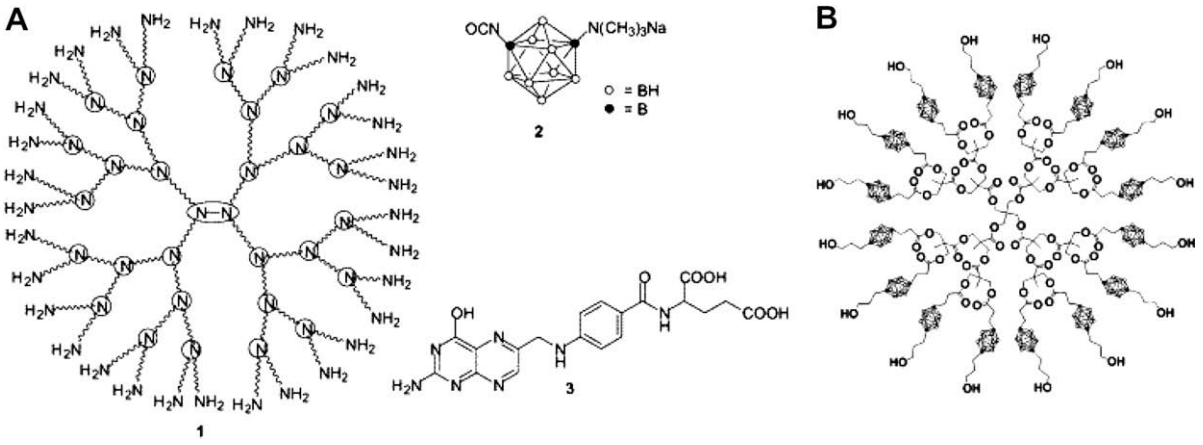

Fig. 12. (A) Schematic presentation of a PAMAM dendrimer G3 (1), boron carrier $Na(CH_3)_3NB_{10}H_8NCO$ (2), and targeting ligand folic acid (3). (B) Incorporation of multiple carborane cages within an aliphatic polyester dendrimer. Reproduced with permission from [89,90]. © 2003 and 2005 American Chemical Society.

24JK-FBP tumors expressing the folate receptor, it also led to an increase in uptake of the dendrimers by the liver and kidneys [89]. In an alternative approach to surface functionalization with carborane, the incorporation of multiple *p*-carborane cages within aliphatic polyester dendrimers was accomplished through the preparation of a bifunctional carborane synthon. Internal dendrimer functionalization is advantageous, allowing peripheral hydrophilic groups to impart aqueous solubility and to effectively mask the presence of hydrophobic carborane cages within the macromolecules. A *p*-carborane derivative having acid and protected hydroxyl functionalities was found to efficiently couple to peripheral hydroxyl groups of low-generation dendrimers under standard esterification conditions. Deprotection of carborane hydroxyl groups allowed further dendrimer growth through a divergent approach, leading to dendrimers G4 and G5 that contain 4, 8, and 16 carborane cages within their interior. These structures exhibited aqueous solubility after deprotection of the surface hydroxyl groups as long as a minimum of eight hydroxyl groups per carborane were present (Fig. 12). Irradiation of these materials with thermal neutrons resulted in emission of gamma radiation that is indicative of boron neutron capture events occurring within the carborane-containing dendrimers [90].

4.9. Self-immolative dendritic prodrugs

In attempts to control and in some cases accelerate the release of active materials from dendrimers or to facilitate the clearance of dendrimers from the body, studies have been conducted to trigger the disassembly of dendritic prodrugs, also coined 'cascade-release dendrimers' and 'self-immolative dendrimers' [91–93]. These unique structural dendrimers can release all of their outer branch units through a self-immolative chain fragmentation, initiated by a single cleavage at the dendrimer's core ('dendritic amplification'). Incorporation of drug molecules as these outer branch units and an enzyme substrate as the trigger can generate a multi-prodrug unit that will be activated with a single enzymatic cleavage (Fig. 13). The first-generation of dendritic prodrugs with doxorubicin and camptothecin as branch units and retro-Michael focal trigger, which can be cleaved by the catalytic antibody 38C2, has been reported. Bioactivation of the dendritic prodrugs was evaluated in cell-growth inhibition assay with the Molt-3 leukemia cell line in the presence and absence of antibody 38C2. A remarkable increase in toxicity was observed. Dependent on the linker molecule, different numbers of drug molecules can be released in one single activation step [94,95].

A similar approach of controlled dendrimer dissociation was applied to enhance the activity of a dendritic fluorescence probe. Targeted fluorescent probes are valuable tools in molecular imaging. Their efficiency as imaging agents can be improved if their fluorescence emission can be turned on only at the target site. Equally important, enzyme targeting can be used to reveal tissue-specific molecular information by imaging. The multiple antigenic peptide (MAP) system is an example of a discrete, dendritic scaffold, which was used to design molecular probes that fluoresce only after enzymatic treatment. The probes incorporated a cathepsin S dipeptide substrate (Leu-Arg) and a PEG chain in their branches (Fig. 14). The fluorescence emission of near-infrared fluorochromes attached to the N-termini of the branch arms was quenched due to formation of H-type dye aggregates within the MAP system. By varying the length of the PEG chain, three probes were synthesized, CyPEG-1, CyPEG-2, and CyPEG-3, with 4, 8, and 12 ethylene oxide units. CyPEG-2 showed optimum aqueous solubility and quenching efficiency for imaging applications. Upon proteolytic activation with cathepsin S, CyPEG-2 showed greater than 70-fold increase and more than 95% recovery in fluorescence emission. MAP-based probes may prove to be a universal and robust design that can be exploited for development of fluorogenic probes for a variety of proteases via alteration of the enzyme substrate in the dendritic arms [96].

5. Conclusions

The high level of control over the dendritic architecture makes dendrimers ideal carriers in drug delivery applications. Small organic drug molecules with diverse structures have been success-

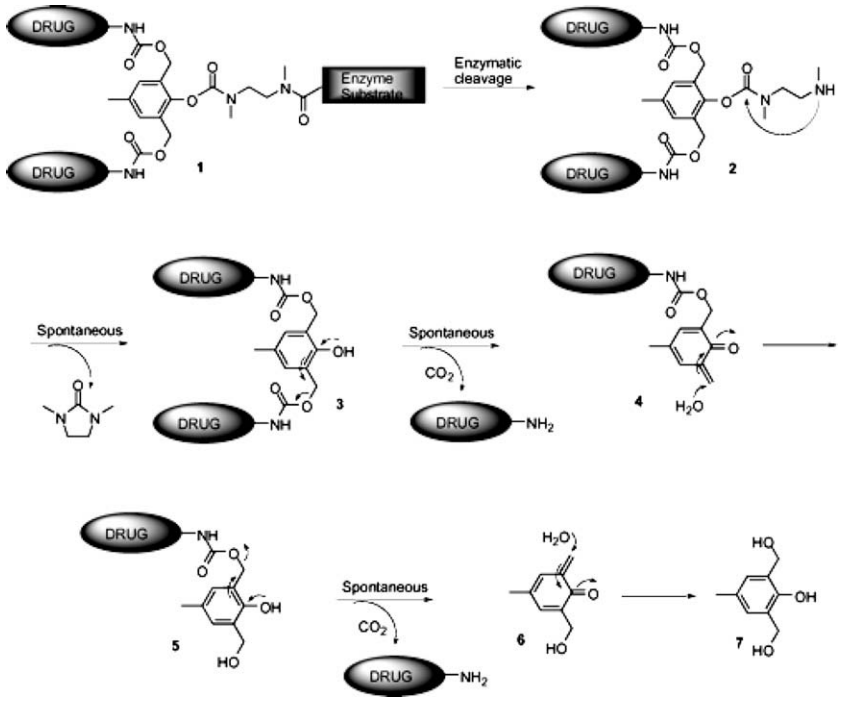

Fig. 13. Mechanism of dimeric prodrug activation by a single enzymatic cleavage. Reproduced with permission from [94]. © 2004 American Chemical Society.

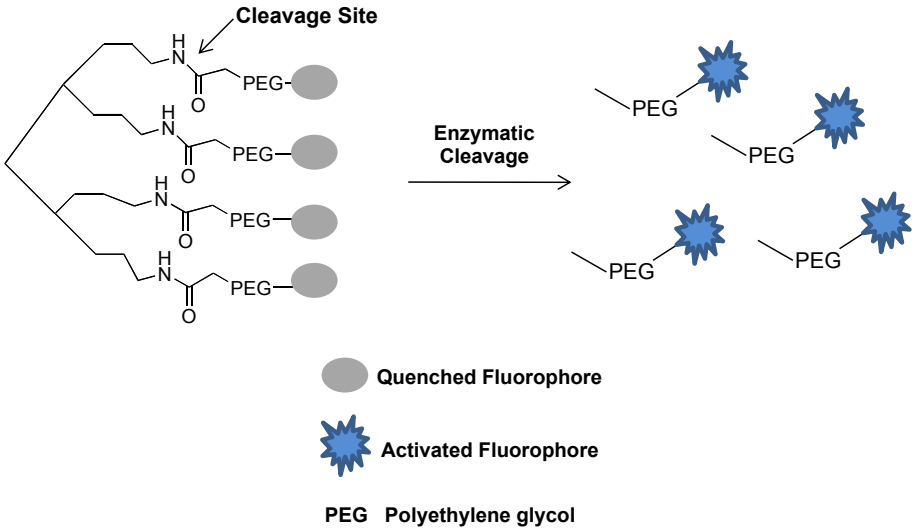

Fig. 14. Design of a fluorescent probe with a molecular switch on a multiple antigenic peptide (MAP) core. Adapted from [96]. © 2006 American Chemical Society.

fully formulated with dendrimers, either through physical association with the dendrimer surface and/or interior, or through chemical conjugation between drug and dendrimer surface. Most studies have focused on two dendrimer families, poly(amidoamine) (PAMAM) and poly(propylene imine) (DAB or PPI) dendrimers. Two promising new families of dendrimers have been developed, poly(etherhydroxylamine) (PEHAM) dendrimers that provide internal functionalities for additional chemical conjugation of drug molecules ('internal prodrug approach'), and dendrimers based on 1,3,5-triazene building blocks. The therapeutic areas for dendrimer–drug formulations include anticancer, anti-inflammatory, and antimicrobial treatments. In many cases, dendrimers have been functionalized with poly(ethylene glycol) (PEG) chains in order to enhance their container properties and to improve the biocompatibility of these carrier molecules. Parameters controlling these features are PEG chain length and their grafting density on the dendrimer surface. The biocompatibility of dendrimers follows patterns known from other small particles such as micelles and liposomes. Cationic surfaces show cytotoxicity; however, derivatization with fatty acid or PEG chains, reducing the overall charge density and minimizing contact between cell surfaces and dendrimers, reduces these toxic effects.

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