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Review

Biofunctional nanosystems based on dendritic polymers

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ABSTRACT

Among the various polymeric architectures, dendritic polymers have received a substantial scientific focus for their highly branched, multifunctional, and well-defined structures. Dendritic scaffolds have found many applications for designing nanoscale drug/gene delivery carriers and constructing diagnostic and biosensor devices, and protein-resistant surfaces. A significant number of research groups across Europe share the common objective, yet in conspicuously individual ways, of utilizing such polymers for devising innovative biomedical tools and techniques. This review describes the European effort of finding the application of dendritic polymers as advanced generation therapeutics within the purview of nanomedicine.

Contents

1. Introduction

Multiple interdisciplinary research groups across Europe are engaged in designing biofunctional nanosystems based on dendritic ('treelike') macromolecules.[1] Even after four decades since their inception, dendritic structures and their built-in nanoscopic and multifunctional features are still receiving considerable scientific interest. Set-off from classical structural chemistry, dendrimer research has now stepped into the domain of applied research in biology, material/engineering sciences, and especially in the area of nanomedicine. Biomedically, dendritic polymers have extensively been studied for drug/gene delivery, designing protein resistant surfaces, fabricating diagnostic agent, and constructing biosensor devices. As synthetic innovation continues to create novel dendritic structures and dimensionality, application areas of these molecules are continually opening up for new and exciting biomedical usage. In this review, we provide a general impression of the trends and tendencies in the European scientific community for using dendrimers in the field of drug delivery and biomedicine. Generally the term "dendritic polymers" is used to denote the family of structurally branched molecules covering dendrons, dendronized polymers, perfect dendrimer and hyperbranched polymers (Fig. 1).

In a dendritic molecule, the modular arrangement of branches confers a two-fold structural parameter which is critical for drug delivery applications. With increasing numbers of generations, the molecule takes on a 3-D spherical shape due to symmetrical congestion of the branching units, thereby yielding supramolecular void spaces and, furthermore, multiple surface functional groups which render the

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[1] Many of these research groups mentioned in this review are members of the European COST program 'Dendrimers in biomedical applications'.

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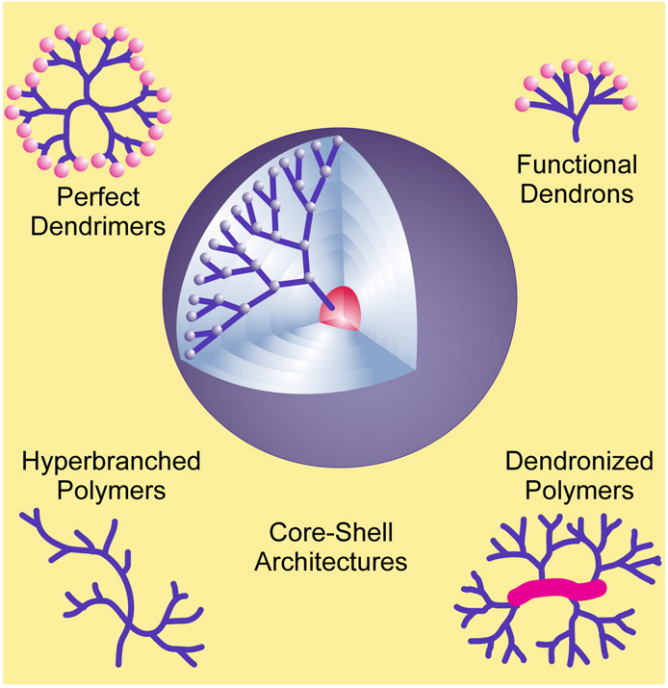

Fig. 1. Schematic classification and nomenclature of dendritic polymers. Reproduced with permission from [1]. Copyright 2004, John Wiley and Sons.

molecule amenable to a wide range of chemical modifications [2]. These two features have particularly made dendritic polymers suitable for delivering bioactive guests either by supramolecular interaction within the structural voids or by direct chemical conjugation to peripheral functionalities. In both cases, it is possible to generate drug delivery vehicles, where the active compounds are homogeneously distributed within the defined dendritic nanosystem, and the complex/conjugate is stable enough to withstand the fluctuating in-vivo milieu. The presence of multiple, terminally active groups also endows the molecule with a 'multivalent' capacity to interact with different cellular components, which is one of the underlying features of their usage as a biological scaffold.

The European endeavors towards the synthesis and biological application of dendritic polymers proceeded after the pioneering report of Vögtle in 1978 featuring the design of dendritic architectures by a divergent strategy [3]. In this methodology, polyfunctional cores were allowed to react with monomer units that have one reactive site and multiple protected or unreactive groups. Vögtle's route typically yielded dendrimers with a AB_2 or AB_3 branching motif, although the strategy was partially hindered by incomplete reaction at higher generations that led to branching defects [4]. Subsequently, there was a surge of structurally diversified and well characterized dendritic architectures with both aliphatic and aromatic backbones [5,6]. Majoral's phosphorus containing dendrimers, van Koten's silane dendrimers, Hult's polyester dendrimers, Meijer/Mülhaupt's poly(propylene imine) dendrimers, and Denkewalter's poly(L-lysine) (PLL) based structures are some examples of compositional diversity that have been realized in the effort to develop structurally novel dendritic molecules [5–10]. Haag and Frey's polyglycerol (PG) based dendritic architectures are exceptional as they stand out as an interfacing molecule between perfectly branched dendrons/dendrimers and hyperbranched polymers [11,12]. Glycodendrimers of Lindhorst, Haag, and Seeberger also show the structural diversification that can be attained within dendritic structures from biologically active starting materials [13–15]. Schlüter's dendronized polymer has been a leading approach for synthesizing hybrid structures where dendrons are attached to a linear polymeric backbone [16]. Most of these structurally diversified molecules have been post-synthetically modified for biomedical application either to deliver drug/genetic materials into systemic circulation or to interfere with specific biochemical and physiological events.

2. Why dendritic polymers?

The unique supramolecular and interfacial features of dendritic polymers, coupled with chemically modifiable functional groups on their terminal branching units, have made them suitable nanocarriers for bioactive agents. As a result, they have been used to design nanoscale delivery systems for drug, genes, diagnostic dye, and biologically active metal ions [2,17–21]. In these molecular delivery systems, the active agent is either supramolecularly complexed within the dendritic scaffold (Fig. 2a) or is covalently conjugated to the terminal functional groups located on the branching units (Fig. 2b). In the case of supramolecular complexes, the guest molecules physically interact with dendritic scaffolds by non-covalent secondary binding processes, for example, by hydrogen bonding, van der Walls interactions, and electrostatic attractions between oppositely charged species located within the drug and the carrier. Dendritic polymers with pharmacologically active end groups such as sulfates and phosphates have also been designed to electrostatically interact with negatively charged cellular components in order to interfere with a particular biochemical event (Fig. 2c). Another main reason for the use of dendrimers in biology and medicine is the multivalency, caused by the presence of a large number of surface end groups within the same molecule that can interact with biological receptors or with other chemical entities to a greater extent and thus generate hierarchical complexes with high binding affinities. These multivalent interactions are considerably stronger than the individual bonding of a corresponding number of monovalent ligands to a multivalent receptor and they are the most predominant phenomena in biological systems, particularly for recognition and attachment, signal transduction, and numerous cellular interactions. Evidence of such multivalent affinity enhancement has been studied in great detail by Lee et al. who proved that the presence of a specific equatorial hydroxyl group located near intersugar glycosidic linkage enhances the binding affinity of viscumin (the major lectin in mistletoe extract) with galactose specific ligands by extending the binding area and "ligand-clustering" effect [22]. Thermodynamically, the driving potential of a multivalent interaction is the high entropic gain in the formation of a multivalent complex. Dendritic scaffolds can easily mimic the so-called "ligand clustering" effect due to the vicinal presence of active functional groups within an adjustable distance. The nanoscale size of dendrimers (typically 1–20 nm) offers a number of structural advantages over other types of micro- and macroscopic drug delivery systems which includes rapid cellular entry, reduced immune activation, and targetability. However, the molecular size and surface properties of dendrimers greatly influence the clearance kinetics and immune activation pattern of the drug-dendrimer complex/conjugate. While dendrimers with the size range of 1–5 nm are rapidly cleared by renal or splenic filtration, larger sized dendritic species are more prone to activate the complementary components of the immune system [23]. As a drug delivery system, dendritic molecules also afford protection to the guest molecule from

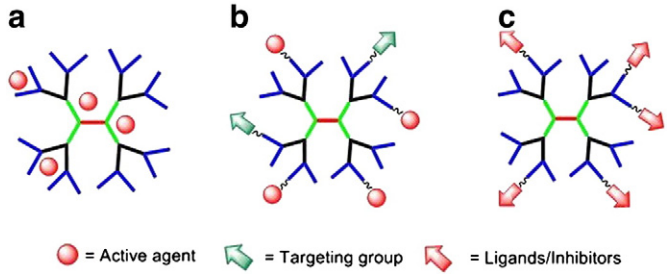

Fig. 2. Different ways to use dendritic polymers as drug delivery scaffolds: (a) non-covalent/supramolecular encapsulation, (b) dendritic polymer conjugates along with targeting groups, and (c) multivalent dendritic polymers (presenting ligands or inhibitors).

systemic degradation and elimination, thereby providing extended plasma residence time of the active drug in the body. For dendritic molecules, interaction with physiological components, which directly governs the biocompatibility and toxicity of the carrier, is adjustable through modulation of the structural motifs. Such structure-dependent activity/toxicity control has given dendritic polymers a prominent position in the class of effective drug-delivery scaffolds [24,25].

3. Drug/dye delivery with dendritic polymers: non-covalent encapsulation within supramolecular architectures

The supramolecular sites within a dendritic polymer can non-covalently harbor drugs, dyes, diagnostic agents, and other biologically active species. The guest molecules can stabilize themselves within these coordination pockets by assembling with the branching fragments of the host through secondary interactions (hydrogen bonding, electrostatic interactions, dipole-dipole, and hydrophobic interactions). It is also possible to attach environment-responsive modalities within such dendritic structure that will facilitate drug release spatially and temporally (Fig. 3). Such spontaneous supramolecular self-assembly enables the utilization of dendritic architectures as drug delivery vehicles and offers several distinct advantages such as enhanced solubilization of non-polar drugs in aqueous media and minimization of non-specific interactions of the complexed drug with plasma components.

Meijer's prototypical work in the early 90s on the synthesis and characterization of so-called 'dendritic boxes' within poly(propyleneimine) dendrimers surrounded by a dense shell is one of the major concepts that was utilized later for 'supramolecularly-encapsulated' drug delivery applications of dendritic polymers [26,27]. Unfortunately but the initial approach was based on a system that was not water-soluble. Subsequently several dendritic molecules have been used as water-soluble supramolecular carriers for a wide variety of biologically active species with substantial therapeutic and pharmaceutical advantages, several of which are illustrated in Fig. 4.

Following this facile supramolecular encapsulation approach, Wheate et al. non-covalently complexed a highly potent anticancer drug, aquated cisplatin with half-generation PAMAM dendrimers in an attempt to design dendrimer-based cancer therapeutics [28]. The amount of drug bound was found to proportionally increase with dendrimer generation and the complex was found to be a releasing system for cisplatin. In vivo activity was examined using an A2780 tumor xenograft. The G6.5 cisplatin–dendrimer complex was administered in two doses (6 and 8 mg/kg equivalent of cisplatin) both of which were well tolerated by the mice. The lower dose displayed comparable activity to cisplatin with a tumor volume reduction of 32%, but the higher dose was significantly more active than free cisplatin with a tumor reduction of 45%, showing the retention and even enhancement of cisplatin activity when complexed within a dendritic scaffold. Wołowiec's group mainly focused on the solubility and in vitro transdermal diffusion of active drugs with the aid of a dendritic structure. PAMAM dendrimers of full and half generation were used by Wołowiec as solubility enhancers of weakly soluble

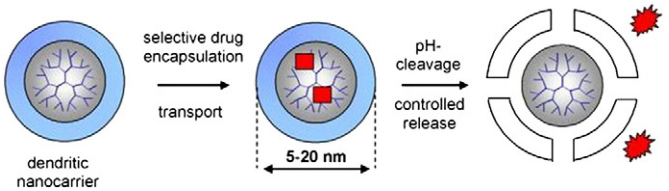

Fig. 3. Unimolecular dendritic nanocarriers for supramolecular encapsulation of biologically active compounds. Controlled release after triggered shell cleavage (e.g. pH-controlled). Copyright © 2004 WILEY-VCH Verlag GmbH & Co.

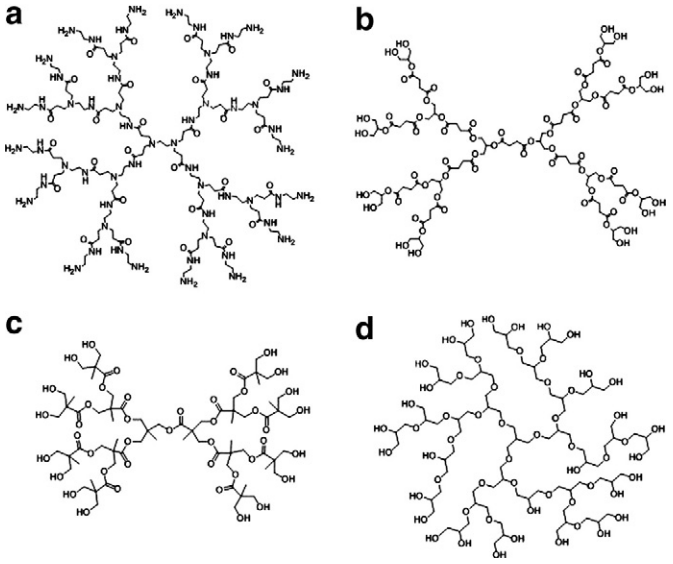

Fig. 4. Examples of several dendritic scaffolds extensively studied for potential drug delivery applications: (a) PAMAM, (b) poly(glycerol-succinic acid) dendrimer, (c) Boltorn® and, (d) hyperbranched polyglycerol.

riboflavin (B_2 vitamin) in methanol. The dendritic molecules were found to weakly enhance the solubility of B_2 (7.2–10.3 times) according to the order: G2 ≫ G2.5 > G3 ≫ G3.5 > G4 while facilitating the permeation of B_2 through simulated membranes according to the order: G2 > G3 ≫ G2.5 > G3.5 > G4. Their work clearly shows the generation–activity relationship when an active molecule is encapsulated within dendritic molecules [29]. The same group has also used PAMAM dendrimers as solubilizers and hosts of 8-methoxypsolarene, thereby enabling the transdermal diffusion of the guest [30]. Kissel et al. has reported nanoparticle formulation from three different hyperbranched polymers, namely, an unmodified dendritic polyester (Boltorn H40™), a lipophilic, fatty acid modified dendritic polyester (Boltorn U3000™), and an amphiphilic dendritic polymer (Boltorn W3000™) for delivery of paclitaxel based on self-assembled encapsulation principle [20].

Dendritic polyglycerol (PG) developed by Haag et al. has proven to be a very attractive and versatile scaffold for drug delivery applications. For supramolecular encapsulation and controlled release of drug and dye molecule, the Haag group generated a library of intelligent core/shell structures based on hyperbranched PG. In one of these scaffolds, a PEG shell was attached to the hyperbranched core through a pH-labile imine-based linker which was designed to cleave off at around pH 5.0 [31]. The dendritic nanocarriers were fabricated by attaching tri-PEGylated benzaldehydes of varying lengths to the hyperbranched PG amine by using pH-labile imine bonds. The designed structures were able to encapsulate the anticancer agent doxorubicin and a near-infrared (NIR) imaging dye indotricarbocyanine. In the latter case, the dye complex was localized selectively in tumors which was demonstrated by NIR-fluorescence imaging of tumor-bearing F9 teratocarcinoma mice. For a more universal encapsulation and transport behavior, the Haag group developed a novel kind of core multi-shell nanoparticles (CMS NPs), initially with a poly(ethylene imine) core and later with a PG core. This unique architecture was inspired by the molecular mimicry of a liposome. In such structures, the hyperbranched PG core was surrounded by double-layered shells (Fig. 5, top panel) [32,33]. The universal supramolecular-entrapment characteristics of the obtained CMS nanotransporters was confirmed by encapsulation of polar guest molecules as rose bengal and congo red, and water insoluble compounds, such as nimodipine, nile red, and pyrene by the same nanocarrier. The CMS NPs exhibited an extremely low critical

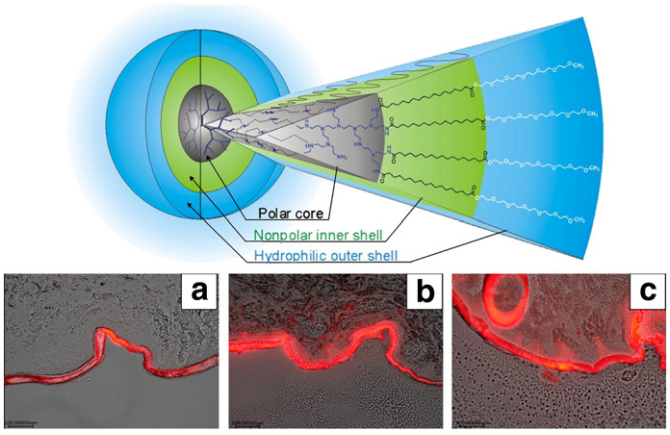

Fig. 5. Core multi-shell (CMS) nanocarrier based on hyperbranched polyglycerol (top panel). Rhodamin B penetration into pig skin: (a) staining of pig skin following the application of 0.004% rhodamin B loaded cream, (b) solid-lipid nanoparticles, and (c) CMS architecture for 6 h (bottom). Copyright 2007, John Wiley and Sons and 2009, Elsevier.

aggregation concentration (CAC, in the order of 10^{-7} M) which indicates that they do not transport as unimolecular carriers. Instead the nanoparticles assemble into highly stable supramolecular aggregates which are responsible for their transport ability. It was found that the aggregate size depended on the geometry of the guest molecules, and the property of CMS NPs to aggregate prior to encapsulation provided the system with a unique feature for their use as a drug delivery vehicle. With rhodamine B loaded as a model guest molecule, the CMS NPs exhibited a higher skin penetration depth than conventional creams and solid-lipid nanoparticles when applied onto porcine skin model (Fig. 5, bottom panel).

Micelles and reverse micelles prepared with dendritic polymers for drug delivery applications have also been widely studied in Europe. Representatively, Leroux and coworkers have been involved in the formation of reverse micelles by the atom transfer radical polymerization (ATRP) technique. The group used 4-, 5-, 6-, and 8-arm ATRP initiators to polymerize glycidylmethacrylate. Hydrolysis of the resulting polymers and partial esterification of the pendant hydroxyl functions with acyl chlorides (12–18 carbons) resulted in the formation of inverted micellar structures [34]. The alkylated scaffolds were shown to self-assemble into reverse micelles (RMs) with diameters in the range of 20–60 nm in organic solvents and/or oil. These derivates were able to encapsulate different water soluble anionic dyes (congo red, brilliant blue R, methyl orange) and various proteins including vasopressin, myoglobin, and albumin in organic solvents and ethyl oleate. A significant enhancement in the loading of model solutes in poly(ester) particles was obtained after the addition of the RMs. As a specific example, the RMs were embedded into poly(D,L-lactide) nanospheres to serve as a hydrophilic reservoir for polar guest molecules. By enhancing the concentration of RMs within the nanoparticles, improved encapsulation efficiency of nile red, congo red, methylene blue, methyl orange, and vasopressin was observed [35]. In another approach the stability of the self-assembled micelles was improved through a cross-linking reaction between the hydroxyl groups located within the micelles [36]. Compared to the non-cross-linked analog, the capacity to retain guest molecules was increased by this structure, although this occurred at the expense of the loading capacity. Leroux's work shows the significance of self-assembly generated supramolecular structures for designing sustained drug delivery or diagnosis systems.

Supramolecular complexes that can release the guest molecule upon environmental stimuli have also been investigated to a great extent. Paleo's earlier systems of acid- and salt-triggered multifunctional scaffold derived from diaminobutane poly(propylene imine) dendrimers (DAB) displayed poly(ethylene glycol) chains and guanidinium moieties on its exterior. This system was found to be specifically suitable for encapsulation and release of pyrene. The guest molecule was released from the scaffold through protonation and/or change of osmotic environment [37]. Paleos has also prepared multifunctional hyperbranched polyether polyols bearing protective poly(-ethylene glycol) (PEG) chains with or without the folate targeting ligand at their termini. They have assessed the solubilization, encapsulation, and osmotically triggered release of a fluorescent probe and tamoxifen [38]. The group has also reported the preparation of an insulin controlled release system with second generation biodegradable poly(L-lysine) dendrigrafts functionalized with 12–48 arginine endgroups. The scaffold interacted at physiological pH with insulin affording dendrigraft/insulin complexes [39].

Supramolecular encapsulation of diagnostic agents within dendritic scaffolds has been an extensively studied field in dendrimer research. The focus of this effort mostly involves delivery and localization of diagnostic agents to target tissues and enhancement of circulation life-time of the diagnostically active species. Extensive research has been carried out on the lanthanide-classed ion Gd (III), which is the most prominent metal ion for magnetic resonance imaging (MRI) contrast agent mostly due to its very high magnetic moment. The interaction between Gd (III) with protons from water molecule lead to high relaxivity value of water protons, which helps generate better contrast images. As the ion is very toxic, a stable Gd (III) complex with diethylenetriaminepentaacetic acid (DTPA) is generally synthesized which is termed as Gd-DTPA. Further reduction of Gd (III) toxicity can be achieved by encapsulation of Gd-DTPA within a dendritic scaffold [40]. To this end, Meijer group designed different generations of Gd(III)DTPA-terminated poly(propylene imine) dendrimers with increasing generation. They found that the magnetic relaxivities non-linearly increased with the molecular weight of the dendritic carriers and a stronger and prolonged blood signal enhancement for the higher generations of the dendritic contrast agent [21]. Furthermore, the company Schering (now Bayer Healthcare) developed a multifunctional Gd-dendrimer imaging probe for MRI imaging which has shown better contrast in clinical phase III studies than the commercial monofunctional Gadovist® [41–47]. Recently, commercially available hyperbranched aliphatic polyester has been used to prepare novel multifunctional gadolinium complexes bearing protective PEG chains, a folate targeting ligand, and EDTA or DTPA chelate moieties. Their relatively high water relaxivity values coupled with biodegradability of the hyperbranched scaffold and folate receptor specificity rendered these non-toxic dendritic polymers promising candidates for MRI applications [48]. Fernandez-Megia and Riguera's group are also heavily involved in exploring their “GATG” (Gallic acid-tetraethylene glycol) dendritic structure for its potential applicability as an MRI contrast agent [49,50].

4. Covalent drug/dye delivery with dendritic molecules: dendritic polymer conjugates

Peripheral end-groups of a dendrimer can be used as attachment points to couple anticancer drugs to the core scaffold. Following Ringsdorf's idea of polymer-drug conjugates [51], not only the active species, but also the targeting fragment and/or solubulizing moiety can be linked to the dendritic carrier molecule to create a multifunctional drug delivery platform. Classically, the key principle for designing such a conjugate lies in the fact that multiple copies of the same or different drug molecules can be attached to each dendrimer molecule by several bioconjugation reactions between the orthogonal and complementary functional groups of the two species. Release kinetics of the active agents from the dendrimer is largely governed by the chemical linkage by which the drug is coupled (Fig. 6). In most cases, the linkage is of an ester or amide type however, a wide and innovative

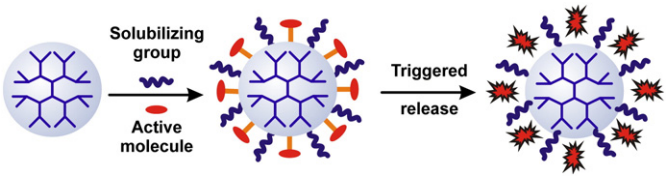

Fig. 6. Design of a dendritic conjugate by attaching multiple copies of drugs and solubulizing groups to the scaffold. Environment-responsive linker system can facilitate triggered-drug release. Copyright 2010, Elsevier.

range of linking chemistry has always been used. In addition to ester or amide types, drugs are also linked to the dendritic scaffold through hydrazone, imine, carbamate, disulfide, carbazate bonds, and enzymatic cleavable peptide sequences. Each of the linkage modules has their inherent, and of course, differential mechanism of cleavage modalities that separate the drug molecule from the carrier scaffold. While ester, hydrazone, and carbamates depend on environmental pH to collapse, amides and peptidic sequences require enzymatic involvement for their degradation and subsequent release of the active species.

Early research in the field of drug-polymer conjugate involved Duncan's G3.5 polyamidoamine (PAMAM) dendrimer with a sodium carboxylate surface which was conjugated to cisplatin, thereby giving a water soluble dendrimer–platinate system which slowly released platinum in vitro (Fig. 7). The dendrimer–Pt conjugate was also less toxic (3- to 15-fold) than cisplatin and thus showed potential for further investigation as a novel antitumor approach [52]. The same group used anionic, polyamidoamine, G3.5 dendrimers to make novel water-soluble conjugates of D(+)-glucosamine and D(+)-glucosamine 6-sulfate with immuno-modulatory and antiangiogenic properties, respectively [53]. Duncan, in collaboration with Veronese et al., has reported the synthesis, characterization, and cellular uptake of novel monodisperse PEG-dendrons. As poly(ethylene glycol) (PEG) is widely used for protein and drug conjugation, the aim of this study was to synthesize for the first time a novel, branched PEG-based architectures, to define their cytotoxicity, and to later profile their cellular uptake. Representative structures were not found to be toxic towards an endothelial-like cell line (ECV304) at concentrations up to 4 mg/ml for over 72 h [19].

Converting an active drug to a polymer conjugated prodrug is a good way to enhance solubility and bioavailability of a wide variety of drug molecules. To this end, D'Emanuele et al. designed dendrimer-based prodrugs of the poorly water soluble model drug naproxen. The drug was conjugated to dendrimers either directly by an amide bond or by ester bonds using either L-lactic acid or diethylene glycol as a linker. In addition, an aliphatic segment of lauryl acid segment was attached to the surface group of G0 PAMAM dendrimer of the diethylene glycol ester conjugate to enhance permeability. In human plasma, the lactic ester conjugate was more stable than the ethylene glycol linked species

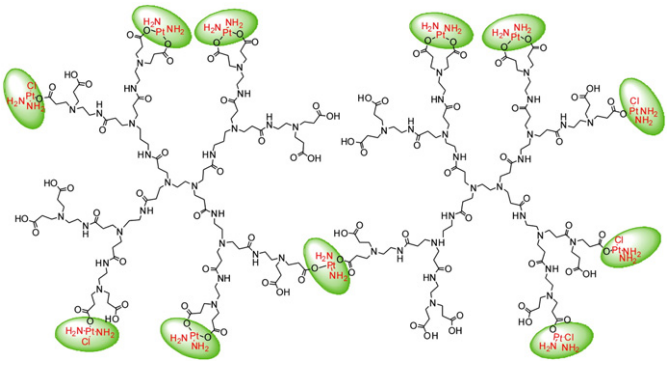

Fig. 7. Schematic representation of Duncan's PAMAM-platinate conjugate where the platinum species is conjugated to terminal branches of PAMAM dendrimer is indicated by the green shaded areas [52].

and the conjugates were non-toxic when exposed to Caco-2 cells for 3 h. Conjugation of naproxen to the dendrimers also enhanced permeability through Caco-2 cell monolayer, and the conjugate with aliphatic unit showed highest permeability [54,55].

Recently, the Haag group developed a straight forward strategy to synthesize thiolated hyperbranched PGs that used a general, flexible method to couple maleimide-bearing prodrugs and diagnostic dyes under physiological conditions in situ. The group reported the use of the thiolated PG scaffold for conjugation to maleimide-bearing prodrugs of doxorubicin or methotrexate which incorporates either a self-immolative para-aminobenzyloxycarbonyl (PABC) spacer coupled to dipeptide Phe–Lys or the tripeptide D-Ala–Phe–Lys as the protease substrate. Both prodrugs are cleavable by cathepsin B, an enzyme which is over-expressed in several solid tumors. The cathepsin B sensitivity of the linking segment is the key modality to release doxorubicin or a methotrexate lysine derivate from the PG scaffold. An effective cleavage of PG–Phe–Lys–DOXO and PG–D-Ala–Phe–Lys–Lys–MTX and release of doxorubicin and methotrexate–lysine in the presence of the enzyme were observed [56]. Cytotoxicity of the conjugates against human tumor cell lines showed that the activity of the drugs was primarily retained. In another investigation Haag et al. prepared a series of conjugates of hyperbranched PG, 10 kDa with (6-maleimidocaproyl)hydrazone derivative of doxorubicin (DOXO–EMCH). The conjugates showed an acid-triggered release of doxorubicin at pH 5.0, and almost marginal release at pH 7.4. The antiproliferative activity against two human tumor cell lines, i.e., AsPC1 LN (pancreatic carcinoma) and MDA-MB-231 LN (mamma carcinoma), showed a 2- to 10-fold lower cytotoxicity profile of the conjugate than the corresponding free drugs. With respect to antitumor efficacy, the conjugates manifested excellent antitumor effects. Using the same synthetic strategy, fluorescent indotricarbocyanin–maleimide was coupled to these scaffolds to follow the cell entry and localization of dendritic PGs.

In another project, the Haag group synthesized thiolated polyglycerol, the scaffold upon which DOXO-EMCH and PEG-maleimide were coupled in a one-pot process, thereby yielding polyglycerol-doxorubicin conjugate with an add-on PEG-shell (Fig. 8) [57,58]. The conjugate showed pH-triggered release as represented by in vitro stability studies where the release of free doxorubicin was below 5% at pH 7.4 and showed a half-life of below 3.5 h at pH 4.0. With a substantially broad toxicity window and substantial cellular accumulation compared to free doxorubicin, the conjugate demonstrated excellent antitumor efficacy when compared with the free drug at equitoxic dose in the ovarian carcinoma A2780 xenograft model. Though it was reported that a 2×8 mg/kg doxorubicin is the MTD in nude mice models and higher doses as 2×13 mg/kg led to unacceptable toxicity and mortality, an orientating toxicity study with PG doxorubicin showed that the conjugate could be administered at 3×24 mg/kg doxorubicin equivalents without producing mortality or severe body weight loss. With respect to antitumor efficacy, doxorubicin showed moderate antitumor efficacy in whole animal. In contrast, treatment with PEGylated doxorubicin conjugates produced excellent antitumor effects that were statistically significant to the control and doxorubicin treated group with tumor remissions for up to 30 days (Fig. 8).

To utilize the size-dependent passive targeting benefit of polymer therapeutics, Ulbrich et al. designed a new biodegradable star polymer-doxorubicin (Dox) conjugate for anti-cancer therapy [59]. In the conjugates the core formed by poly(amido amine) (PAMAM) dendrimers was grafted with semitelechelic N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers bearing doxorubicin attached by hydrazone bonds, which enabled intracellular pH-controlled drug release, or by a GFLG sequence, which was susceptible to enzymatic degradation. The polymer grafts bearing the active drug were attached to the dendrimers either through stable amide bonds or enzymatically or reductively degradable spacers for intracellular biodegradation and enhanced elimination. Biodegradability tests showed that the rate of

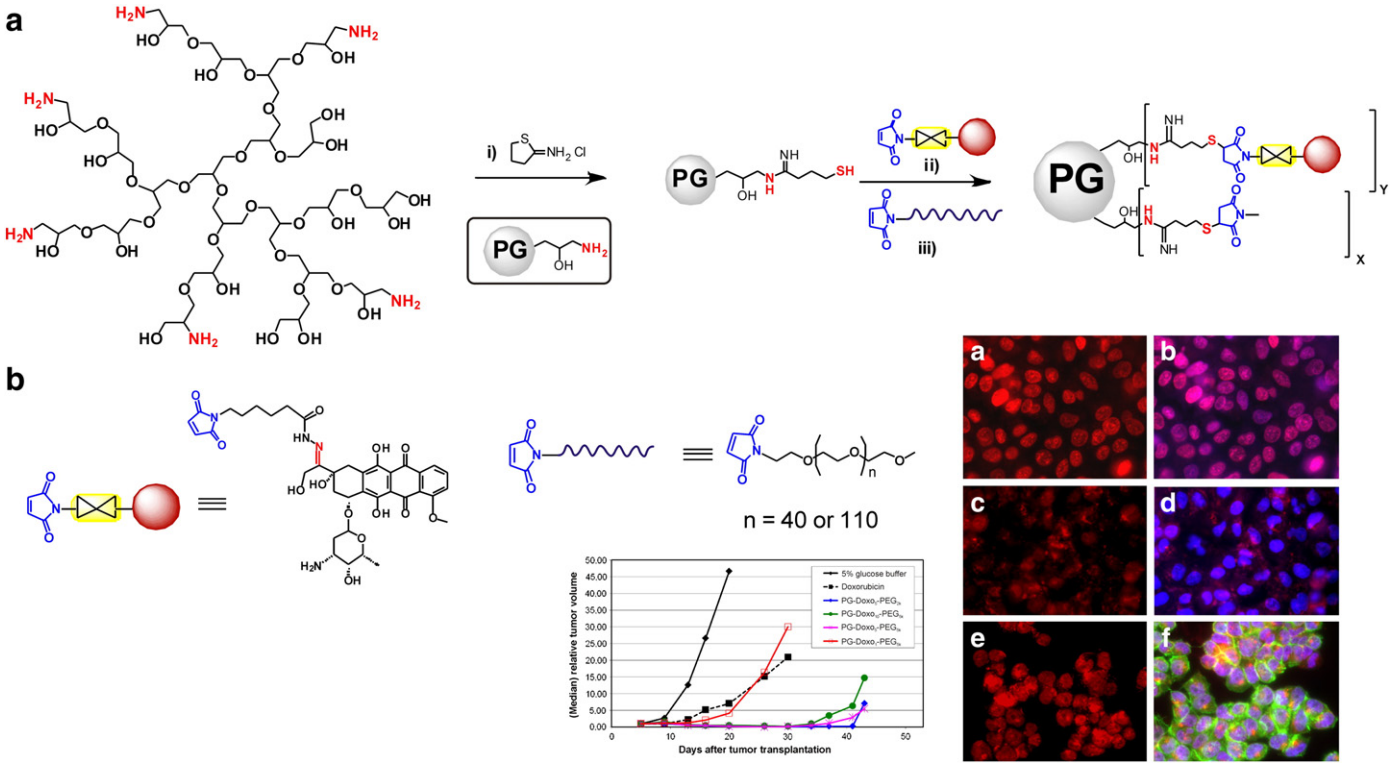

Fig. 8. Schematic pathway for the synthesis of doxorubicin-polyglycerol conjugates with cellular and in-vivo data. Panel 1: synthetic protocol of PG-conjugate: (i) phosphate/EDTA buffer, pH 7.0, r.t., 20 min (ii) phosphate buffer pH 5.8, r.t., 10 min and (iii) phosphate/EDTA buffer, pH 7.0, r.t. 60 min. (a) Schematic representation of amino-bearing hyperbranched PG, (b) (6-maleimidocaproyl-(hydrazone) derivative of doxorubicin (DOXO-EMCH), and (c) PEG-Mal, 2 or 5 kDa. Panel 2: representative fluorescence merge images of A2780 cell incubated at 37 °C with doxorubicin for 4 h (a, b), and PG-Doxo10-PEG5k for 4 h (c, d) or 6 h (e, f). Merge pictures show red (doxorubicin), blue (nuclei stained with DAPI), and pink (colocalization) color. Panel 3: Curves depicting tumor growth inhibition of subcutaneously growing A2780 xenografts under therapy with doxorubicin and the conjugates; PG-Doxo5-PEG2k, PG-Doxo10-PEG5k, and PG-Doxo5-PEG5k were statistically significant to control (day 20) and doxorubicin treated group (day 30); $p<0.05$ (*U*-test). Copyright 2011, Elsevier.

degradation was much faster for reductively degradable conjugates (completed within 4 h) than the degradation of conjugates linked via an enzymatically degradable oligopeptide GFLG sequence (within 72 h), probably due to the difference in steric hindrance for the small molecule glutathione and the enzyme cathepsin B. As for drug release, the conjugates were fairly stable in buffer at pH 7.4 (model of blood stream) as in Haag's system but released doxorubicin either under mild acidic conditions or in the presence of lysosomal enzyme cathepsin B, both of which are abundant in tumor cell microenvironment. Such dendritic and star-like architectures have also been investigated for their distribution and anti-tumor efficacy in EL4 T-cell lymphoma cells [60]. The star polymer-Dox conjugates exhibited significantly prolonged blood circulation and enhanced tumor accumulation in tumor-bearing mice, indicating the important role of the EPR effect in its anti-cancer activity. The star polymer conjugates showed prominently higher in vivo anti-tumor activities than the free drug or linear polymer conjugate when tested in mice bearing EL4 T-cell lymphoma.

Pasut et al. have designed a dendritic structure based on PEG and investigated the role of a heterobifunctional PEG-dendrimer, H_2N-PEG-dendrimer-COOH, as carrier for a combination of paclitaxel and alendronate. The paclitaxel-PEG-Alandronate conjugate was designed to exploit active targeting by the ALN molecule and passive targeting through the enhanced permeability and retention (EPR) effect. Their conjugate demonstrated a great binding affinity to the bone mineral hydroxyapatite in vitro and an IC_{50} comparable to that of the free drugs combination in human adenocarcinoma of the prostate (PC3) cells. The conjugate exhibited an improved pharmacokinetic profile compared with the free drugs owed to the marked increase in their half-life [61].

Not only the drug, but also the targeting fragments are chemically conjugated to the dendritic scaffold as homing units that target a specific tumor milieu. To this end, in vivo selection of phage libraries that display random peptide sequences on their surface has yielded a number of peptides that specifically bind to tumor tissue. In a recent study, two different peptides were introduced to synthetic dendritic scaffolds via oxime chemistry and the resulting compounds were analyzed for tumor homing [62]. The two tumor-homing peptides were developed via in vivo phage display, the first one is a linear peptide consisting of five amino acids (CREKA) and the second one is cyclic nonapeptide, Lyp-1(CGNKRTRGC). The CREKA peptide recognizes the product of blood clotting present in extracellular matrix of tumor and the cyclic nonapeptide specifically recognizes tumor lymphatics, macrophage and tumor cells in hypoxic areas [63–66]. Modification of the dendritic wedge with a short, linear peptide that homed to clotted plasma proteins showed recognition to a specific tumor receptor. However, the extravasation was found to be affected by the size of the construct. In contrast, a positively charged cyclic peptide with cell penetrating properties was capable of directing the entire dendritic architecture toward a specific receptor in tumor lymphatics. These observations are in accord with previous reports for micelles and nanoparticles and emphasize the influence of peptide properties and overall size on the biodistribution of multivalent dendritic macromolecules.

Although, the above-mentioned examples of diversified multifunctional and multi-valent conjugation approaches exhibited substantially positive biological results, most of the drug–dendrimer conjugates suffer a common and serious setback. In an elegant work, Mullen et al. have successfully proved that conjugating different classes of ligand molecules to dendritic carriers results in a heterogenic distribution of ligand-bound dendritic carriers in terms of the number of ligands per carrier. The ligand distribution on a dendritic molecule is dependent on the synthetic history of the carrier molecule, and becomes more

inhomogeneous as the number and type of ligand species and synthetic steps increase [67]. Such a heterogeneous ligand distribution on dendritic surface has indeed serious implications for eventual biological and clinical observations, and may not provide a clear structure–activity picture of the drug–dendrimer conjugate.

5. Polycationic dendritic architectures for gene delivery

Efficacy of gene delivery by polymeric carriers is an important example of biochemical multivalency where manifold copies of the attachment sites enhance the affinity between the guest and the host species. The electrostatic interaction between the negative phosphate backbones of the DNA/RNA strand with dendritic polymers that carry multiple positively charged groups (i.e. dendritic polyamines) is the principle which is used in dendrimer-based non-viral gene delivery approach. The resulting polyplexes (nuclear materials/dendrimer complex) is endsomally uptaken by the target cells, and following lysosomal fusion and subsequent endosomal escape, the genetic materials are released from the complex in the vicinity of nucleus (Fig. 9).

Diversified molecular architectures are attached to dendritic polymers to design efficient gene delivery scaffold. The interaction between a single protonated amine and the phosphate backbone of DNA is relatively weak and competes with salt binding under biological conditions. Therefore tetra-amines such as spermine are used to achieve DNA binding [69]. To achieve several co-operative interaction sites for genetic materials, polycationic dendritic scaffolds can be an attractive alternative. Paleos et al. designed guanidinylated PPI dendrimers which formed well characterized dendriplexes with plasmid DNA and systemically displayed a structure–transfection relationship with cytotoxicity [70]. The Paleos group has also shed some light on the structure–membrane permeation rapport of such systems [71,72]. With hyperbranched dendritic systems, they have prepared hyperbranched polyether polyols that were partially functionalized with quarternary or tertiary ammonium groups. The quarternerized polyols showed a superior transfectability of genetic material over tertiary amine functionalized scaffold claimed from destabilization of the lysosomal membrane [73,74].

Kissel's group at Marburg has extensively worked on different polycationic dendritic polyamine architectures and their wide range of applicability for gene transfection [75]. They have carefully focused on the pulmonary delivery of genetic material and have made substantial progress in this field [76]. They also have investigated the applicability of triazine dendrimers as nonviral vectors for in vitro and in vivo RNAi delivery [77]. Before assessing their RNAi efficiency, the group has undergone an extensive testing of the utilization of triazine based dendrimers as a nonviral gene delivery system and elucidated the effect of molecular features of the dendritic scaffold on biological activity [78]. The Kissel group has also reported a series of non-toxic and biodegradable gene carriers, hyperbranched polymers based on 2,2-bis-(methylol)propionic acid (bis-MPA), (Boltorn H) which were amended by introducing tertiary amines. The terminal OH groups were modified with diethylaminopropylamine (DEAPA) by carbonyldiimidazole (CDI) chemistry. These carriers provide degradability, very low toxicity, and the ability to transfect cells as a function of degree of amine substitution [79].

The use of polycationic dendritic architecture as nonviral vectors of gene therapy has also interested the Smith group. This group has reported a series of dendrons based on the Newkome-type dendritic scaffold that displays a naturally occurring polyamine (spermine) on their surface and investigated the ability of these dendrons to transfect DNA into cells as determined by the luciferase assay [80]. Kostianinen, Smith, and Ikkala generated an optically triggered DNA release system from multivalent dendrons by degradation and with charge-switching multivalency, which has recently been an important concept in designing stimuli-responsive drug delivery systems (Fig. 10) [81]. They have also prepared multivalent Newkome-type polyamine dendrons that function as synthetic DNA binding domains, which in turn can be conjugated with proteins. These polyamine dendrons utilize a spermine surface group to bind to DNA with substantial affinity and are attached onto protein surface in a quantitative and site-specific manner to yield well-defined one-to-one protein-polymer conjugates. In this case the use of N-maleimido-core dendrons selectively reacted via 1,4-addition with the free thiol group on the protein surface, which was either Cys-34 of bovine serum albumin (BSA) or engineered cysteine mutant of Class II hydrophobin (HFB1), resulting in BSA- or HFB1dendron conjugates. Ethidium bromide displacement assay showed a high affinity binding of the dendrons-protein conjugate to DNA [82]. Smith's other significant contribution involves the design of novel gene delivery agents based on combining cholesterol units with spermine-

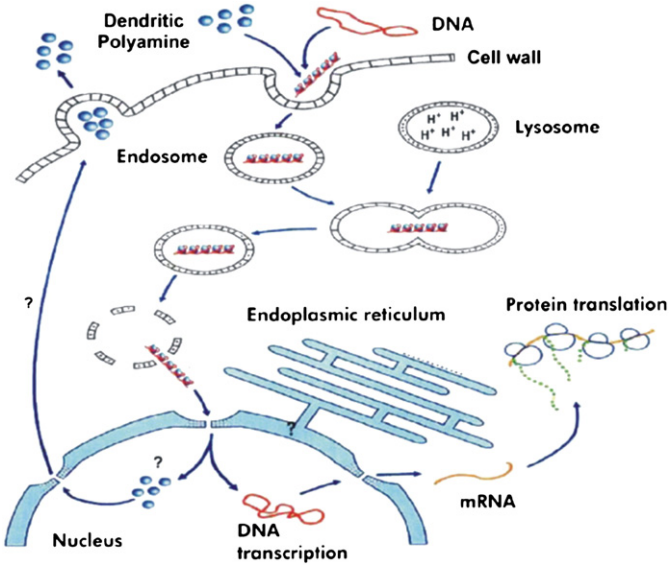

Fig. 9. Mechanism of dendritic nanocarriers for cell-internalization of genetic materials. Adopted with permission from [68].

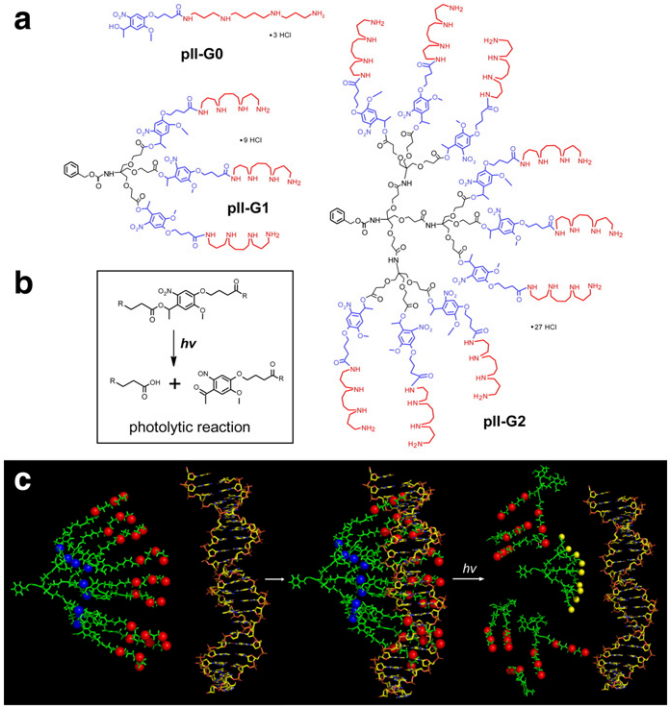

Fig. 10. Smith's photo-cleavable dendritic architecture is able to complex an on-demand release of DNA. Copyright 2007, John Wiley and Sons.

functionalized dendrons [18], interconnected low-generation poly(L-lysine) (PLL) dendrons, [83] and the concept of 'On–Off' multivalent recognition of degradable dendrons with DNA [84].

Recently, the Smith group has developed hydrophobically modified dendrons with a surfactant-like property with hydrophilic spermine surface groups and a variety of lipophilic units at their focal points which can self-assemble and subsequently bind to DNA with high affinity [85].

Haag et al. have synthesized amine terminated PG, the so-termed PG-Amine and several hyperbranched PG derivatives that were post-modified with spermine, spermidine, and pentaethylenehexamine. These dendritic polyamine compounds were clearly multivalent in gene complexation, as observed when the charge excess (CE_{50}) values between monomeric and PG-conjugated amine variants are compared for estimation of DNA binding efficiency. The in vitro gene silencing properties of the synthesized PG-amines were evaluated in human epithelial carcinoma cell (HeLaS3) line with different proteins (Lamin, CDC2, MAPK2). Several pH-responsive core shell architectures that contain the above-mentioned nitrogen shell motif and a polyglycerol core have been designed in a two-step protocol involving the activation of primary and secondary hydroxyl groups by phenyl chloroformate and amine substitution. The most interesting feature of these multivalent PG-Amine structures lies in the fact that the resulting polyplexes yielded similar knockdown efficiencies as the in vitro benchmark HiPerFect® with comparably low cytotoxicity. In contrast to many transfection agents PG-Amine is a promising candidate for siRNA delivery in vivo [86]. The polyglycerol-based cationic dendrimers with core-shell structures developed by Haag et al. also strongly improved the stability of the siRNA, in particular, its intracellular trafficking, silencing efficacy, and accumulation in the tumor environment owing to the enhanced permeability and retention effect. Polyglycerol based dendritic nanocarriers exhibited low cytotoxicity and high efficacy in delivering active siRNA into the cells. With the use of human glioblastoma and murine mammary adenocarcinoma cell lines as model systems, these siRNA-dendrimer polyplexes silenced the luciferase gene, ectopically overexpressed in these cells. Significant gene silencing was accomplished in vivo within 24 h of treatment with Haag's luciferase siRNA-PG-Amine nanocarrier polyplexes, as measured by noninvasive intravital bioluminescence imaging (Fig. 11). Furthermore, the siRNA-nanocarriers showed very low levels of toxicity with the result that no significant weight loss was observed after intravenous administration of the polyplexes [87].

Recently, the Haag group has reported photo-responsive polyglycerol-core shell nanoparticles where the shell is composed either of bis-(3-aminopropyl)methylamine or pentaethylenehexamine derivatives and is attached to the PG core with a photo-responsive o-nitrobenzyl linker. The system was found to release genetic material upon irradiation at a specific wavelength [88]. In comparison to other dendritic structures, the PG based scaffolds have the added advantage of being open and flexible, which is a prerequisite for interaction with genes. Due to the polyether structure, the toxicity remains in a considerably low range.

A series of water-soluble polycationic dendrimers with a phosphoramidothioate backbone (P-dendrimers) were studied in human cell culture by Majoral's group. Preliminary studies have shown that Majoral's phosphorus based dendrimers, with N,N-diethyl-ethylenediamine hydrochloride functions at the surface, show rather moderate cytotoxicity toward HeLa, HEK 293, and HUVEC cells in a standard MTT assay in serum-containing medium. These compounds efficiently delivered fluorescein-labeled ligodeoxyribonucleotide into HeLa cells in serum-containing medium. The dendrimers were found to be successful mediators of transfection of the HeLa cells with a DNA plasmid containing the functional gene of enhanced green fluorescent protein (EGFP) [89].

6. Polyanionic dendritic architectures: designing anti-inflammatory dendritic polymers

Back in 2004 it was reported by Shaunak et al. that polyanionic dendritic structures possess anti-inflammatory properties per se. [90]. A 1,2-diaminoethane-centered G4.5 PAMAM scaffold terminated with 64 carboxylic acid groups, 14% of which was functionalized with glucosamine (MW = 13.6 kDa) and glucosamine-6-sulfate (MW = 14.0 kDa), was found to have an anti-inflammatory effect towards human immune cells. The anti-inflammatory efficacy of glucosamine containing dendritic scaffolds was evaluated by measuring the release of proinflammatory chemokines and cytokines by different immune cells after being inducted by microbial lipopolysaccharide (LPS). The study showed that dendritic scaffolds inhibited the LPS-mediated release of chemokines and cytokines by total peripheral blood mononuclear cells (PBMCs).

In 2006, the Majoral group reported phosphorus-based dendrimers capped by amino-bisphosphonate groups. This azabisphosphonate (ABP) dendrimer, which is presented in Fig. 12, showed high activating

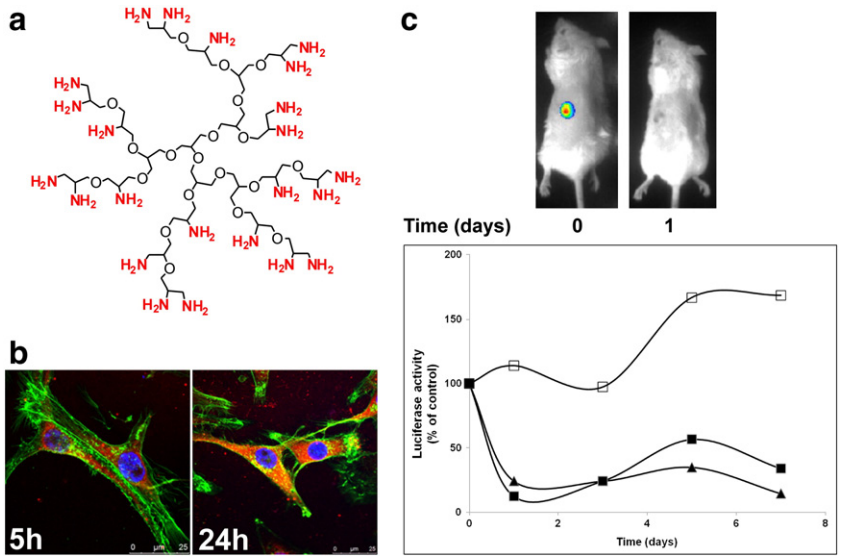

Fig. 11. Illustration of PG-Amine as siRNA carrier: (a) molecular structure of PG-Amine, (b) intracellular uptake of FITC-labeled siRNA complexed with PG-Amine in U87-Luc cells after 5 and 24 h [scale bars of 25 μm], and (c) SCID mice with U87-Luc tumors were treated with 1 mg/kg PG-Amine complexed with 2.5 mg/kg luciferase siRNA (■), 20 mg/kg PG-Amine complexed with 5 mg/kg luciferase siRNA (▲) or saline as control (□). Mice were injected twice on d0 and d4.
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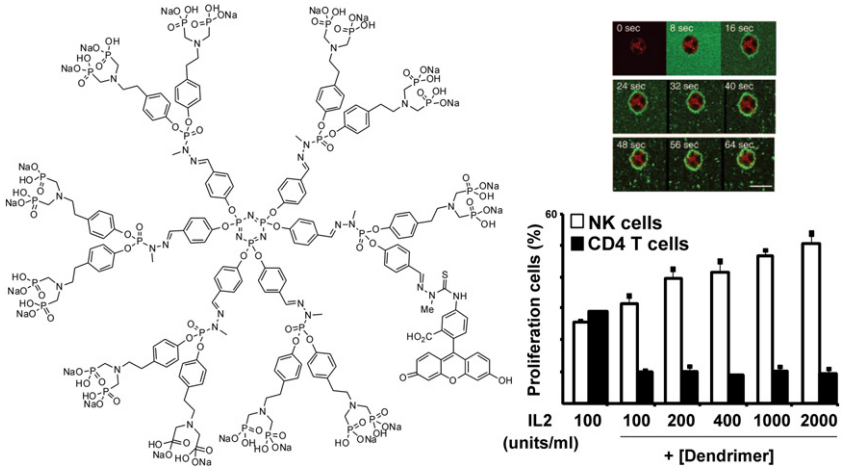

Fig. 12. The ABP phosphorus dendrimer with fluorescent tag (left panel). The inset (top right panel) shows the confocal videomicroscopy of purified monocytes enriched with accumulated fluoro-tagged dendrimer (green fluorescence) in a time-dependent fashion [92]. The graph at the bottom right shows the impaired proliferation of CD4 T cells in the presence of phosphorus dendrimer which is not rescued by higher IL-2 concentration. This indicates a specific inhibition of CD4 lymphocyte compartment by the dendrimer. This inhibition enhances in turn the ex vivo expansion of NK cells from PBMCs for immunotherapy [93].
With permission from American Societies for Experimental Biology, 2006.

properties towards human monocytes [91]. These properties were mainly depicted as changes in the morphology and the phenotype of monocytes, increase of their phagocytosis activity, and their survival in culture at concentrations in the micromolar range.

After screening a wide structural set of phosphonic acid-terminated, phosphorus-containing dendrimers on monocyte activation, Majoral's group found out that the multivalent character and phosphonic acid capping of dendrimers are crucial for monocyte targeting and activation, and are therefore effective inhibitors of the inflammatory process. Confocal microscopy showed that a fluorescein-tagged dendrimer binds to isolated monocytes and is internalized within a few seconds. They also found that dendrimers follow the phagolysosomial route during internalization by monocytes and proved that they could be ideal candidates to develop new drugs for immunotherapies. Majoral's group has also demonstrated the multiplication of human natural killer cells by nanosized phosphonate-capped dendrimers [94] and has also established a protocol for tailored controlling and optimization of the number of phosphonic acid termini on phosphorus-containing dendrimers for the ex vivo activation of human monocytes [95].

As the most important step for activating the inflammatory response is the recruitment of the inflammatory effectors or their precursors from the blood to the site of inflammation, a potential approach to anti-inflammatory therapy is to interfere with leukocyte trafficking [96]. The extravasation of leukocytes through the endothelial barrier to the sites of inflammation is initiated by selectin-induced leukocyte tethering and rolling on the endothelial surface. Selectins are glycoproteins of the lectin family, expressed by both leukocytes (L-selectin or CD62L) and endothelial cells (E- and P-selectins or CD62E and CD62P). To this end, targeting selectins with substantial affinity will be of critical importance in designing successful anti-inflammtory agents.

According to this hypothesis, Dernedde and Haag et al. have investigated dendritic polyglycerol sulfates (dPGS, Fig. 13a) as multivalent polyanionic inhibitors of inflammation that mimicked the mechanism of naturally occurring ligands to the leucocytes. They found that dPGS inhibited both leukocytic L-selectin and endothelial P-selectin with high efficacy where the size and degree of sulfation of the polymer is the governing factor for the selectin binding affinity [97]. As both L-selectin on leukocytes and P-selectin on endothelial cells are interfered by dPGS, the active dPGS should inhibit leukocyte extravasation to inflammatory sites. The proof-of-concept was established in vivo in a mouse model with acute allergic contact dermatitis with typical symptoms of inflammation. Contact dermatitis was induced in mice at day zero by a first application of trimellitic anhydride (TMA) and challenged at day 5 by a second application of TMA in the ears. Before applying TMA, the test compounds (dPGS at 3, 10, and 30 mg/kg, heparin, and prednisolone as a positive control at 30 mg/kg), were injected subcutaneously, and ear-swelling was recorded 24 h later. The experiment showed that both prednisolone (a clinically used glucocorticoid) and dPGS with 61% level of functionalization with sulfate group clearly reduced the ear swelling in a dose-dependent manner for dPGS. The observed reduction in ear swelling by dPGS and prednisolone can be assigned to the reduction of leukocyte extravasation to inflamed ear tissue as the enzymatic activity of neutrophil elastase was dramatically reduced in ear homogenates from mice treated with dPGS or prednisolone control. We recently demonstrated that dPGS acts as a novel type of synthetic nanocarrier and is also suited for inflammation-specific molecular

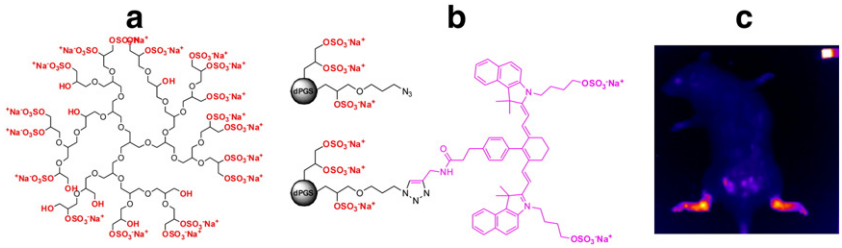

Fig. 13. (a) Idealized structure of dPGS, (b) dPGS conjugated to an fluorescence ICG dye, and (c) In vivo fluorescence imaging of rats with joint inflammation (collagen-induced); i.v. injection of dPGS-dye (2 mg/kg); recorded after 1 h (irradiation: 760 nm).

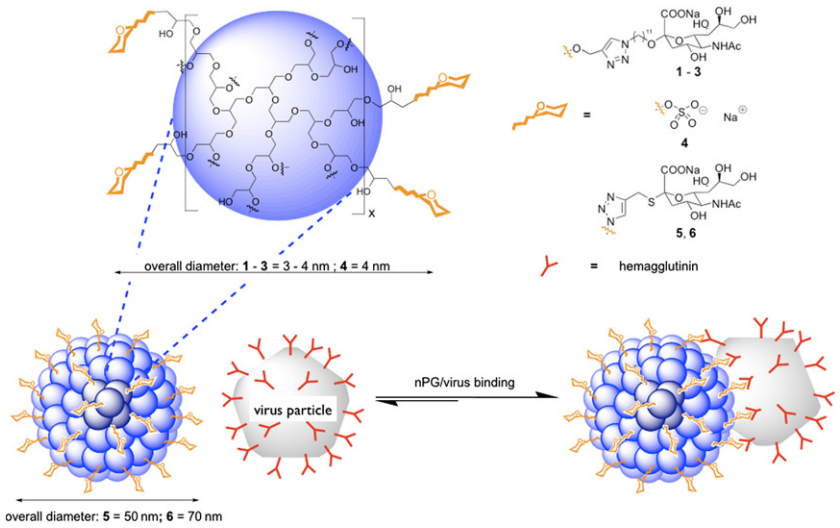

Fig. 14. Sialic acid conjugated dendritic polyglycerol nanoparticles with diameters in the range of 60 nm efficiently block viral infections by polyvalent inhibition of the hemagglutinin in contrast to their smaller multivalent analog. Copyright 2011, John Wiley and Sons.

imaging. Near-infrared fluorescent dye related to Indocyanine Green (ICG) was conjugated to dPGS. Translation into the diagnostic application was accomplished by in vivo fluorescence imaging in a rat rheumatoid arthritis model (Fig. 13b–c), demonstrating fast and highly selective targeting of chronic inflammations [98].

On the basis of the findings that dPGS exhibits high affinity for L- and P-selectin, Haag et al. synthesized other polyanionic macromolecules based on dendritic PG which have been realized by "click-reaction" of polyglycerolazide precursors and alkyne-functionalized anions such as sulfonates, carboxylates, phosphonates, and bisphosphonates. It was later observed that the efficiency of L-selectin inhibition increases in the order carboxylate < phosphate < phosphonate ≈ sulfonate < bisphosphonate < sulfate [99,100]. Along with inhibiting anti-inflammatory events, anionic dendritic scaffold also demonstrates antiviral activity. A series of sialic acid conjugated dendritic polyglycerol nanoparticles with diameters in the range of 50–100 nm was found to possess anti-viral efficacy. Particle sizes varied with the degree of functionalization to match the corresponding virus size and receptor multiplicity in order to achieve maximum efficiency for the inhibition of influenza virus. It has been demonstrated that such polyvalent nanoparticles inhibit influenza A virus cell binding and fusion and consequently infectivity, which indicates the potential of polyanionic dendritic polymers in antiviral therapy (Fig. 14) [101]. Recently a facile preparation of disulfide crosslinked polyglycerol hydrogels was described by Haag et al. where the length scale of polyglycerol particles (3 nm) was extended by preparing nanogels (32 nm) with miniemulsion techniques and microgels (140 and 220 μm) with microfluidics [102]. The structures showed substantial biocompatibility with yeast cell-line and their potential use as matrix and carrier systems for living cells has been predicted.

In this context a number of HIV inhibiting dendrimers are also being investigated. Besides the clinically investigated vivagel® from Starpharma, several anionic glycodendrimers have been reported. However, many of them only interact with individual viral proteins and lack the potential of real polyvalency which requires virus-size dimensions. Therefore these early concepts of dendritic multivalent approaches need to be extended to higher multiplicity and longer length scales to compete with pathogenic viruses or bacteria in the 100 nm to 10 μm range.

From the perspective of glycodendrimers, it is also necessary to mention that a number of groups have engaged themselves in designing and using glycodendrons for biosensor applications since Lindhorst's pioneering effort with hyperbranched glycodendrons and their role as potential inhibitors of type 1 fimbriae-mediated bacterial adhesion in an ELISA [14,103]. Seeberger et al. reported the synthesis of homogeneous, fluorescent and sugar-functionalized metallic dendrimers with varying numbers and types of monosaccharides using a self-assembly process and also have shown their efficiency as lectin sensors by turbidity assays [15,104]. This group also advanced the synthesis of Ru(II)-carbohydrate dendrimers as photoinduced electron transfer lectin biosensors [104,105] and reported the facile synthesis of such Ru(II)-carbohydrate dendrimers via Cu(II)-catalyzed Huisgen-[3+2] cycloaddition [106]. Appelhans et al. also showed the maltose containing poly(propylene imine) dendrimers and demonstrated their exclusive binding properties with ATP molecules through supramolecular recognition. Klanjert et al.'s effort in the field of dendrimers has expanded the understanding of how dendritic molecules interfere with different therapeutically and biochemically active proteins like prion peptides [107] and amyloid fibrils, as well as with liposomal structures representing cellular membranes [108].

7. Conclusion

The focus of this article is to show the major and recent trends of European research in using nanoscale dendritic polymers for innovative aspects of biomedical applications. National, international, and intercontinental support is available which provides substantial logistics to carry out such investigations. Scientific curiosity coupled with a need for novel devices and techniques for addressing clinical demands can be considered as the principle driving force for conducting research on dendritic polymers for biomedical applications. There are still several unmet issues that seriously limit the use of dendrimers for therapeutic applications. Although highly innovative and elegant synthetic pathways are continually being reported in chemical literature for the synthesis of dendritic polymer, most of them cannot be translated in a true sense to an industrial scale, mainly due to the involvement of multi-step fabrication and purification techniques. So far as drug-dendritic conjugates are concerned, the risk of an "average" distribution of drug and/or targeting ligands on dendrimer surface imposes the serious question on the reproducibility and standardization parameters as required clinically. However, through the design of improved synthetic strategies, scalable purification techniques, and an accurate characterization process, the endeavor to place the dendrimer-based therapeutics from laboratory bench to bedside still continues. Enrichment of knowledge in the field of dendritic polymers will indeed stretch the field of drug delivery and targeting towards an advanced level of innovative nanomedicines.

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