CRITICAL REVIEW

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Dendrimers in drug research

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Dendrimers are versatile, derivatisable, well-defined, compartmentalised chemical polymers with sizes and physicochemical properties resembling those of biomolecules *e.g.* proteins. The present *critical review* (citing 158 references) briefly describes dendrimer design, nomenclature and divergent/convergent dendrimer synthesis. The characteristic physicochemical features of dendrimers are highlighted, showing the effect of solvent pH and polarity on their spatial structure. The use of dendrimers in biological systems are reviewed, with emphasis on the biocompatibility of dendrimers, such as *in vitro* and *in vivo* cytotoxicity, as well as biopermeability, biostability and immunogenicity. The review deals with numerous applications of dendrimers as tools for efficient multivalent presentation of biological ligands in biospecific recognition, inhibition and targeting.

Dendrimers may be used as drugs for antibacterial and antiviral treatment and have found use as antitumor agents. The review highlights the use of dendrimers as drug or gene delivery devices in *e.g.* anticancer therapy, and the design of different host–guest binding motifs directed towards medical applications is described.

Other specific examples are the use of dendrimers as 'glycocarriers' for the controlled multimeric presentation of biologically relevant carbohydrate moieties which are useful for targeting modified tissue in malignant diseases for diagnostic and therapeutic purposes. Finally, the use of specific types of dendrimers as scaffolds for presenting vaccine antigens, especially peptides, for use in vaccines is presented.

1 Introduction – Dendrimer types and history

Dendritic structures are found widely in nature. These hyper-branched structures have the advantage that they display a desired motif in a multivalent fashion, in order to give synergistic enhancement of a particular function. Recently, a scientific report on the dry adhesion of Geckos' feet to surfaces interestingly revealed that the Gecko foot is built up by a dendritic network of foot hairs, ending in millions of foot-hairs which create an extraordinary strong adhesion due to multiple van der Waals forces between each foot hair and the surface.[1] In synthetic organic chemistry, dendritic structures emerged in a new class of polymers named 'cascade' molecules, first reported by Vögtle and his group.[2] Later on, development of these molecular designs together with advanced synthetic techniques gave rise to larger dendritic structures,[3–5] and this class of molecules was renamed *dendrimers*. The word dendrimer arises from the Greek *dendron*, meaning 'tree' or 'branch', and *meros* meaning 'part'.[6] Other names for dendrimers are 'arboroles' or 'cascade polymers'. Dendrimers are, despite their large molecular size, structurally well-defined, with a low polydispersity in comparison with traditional polymers. On a molecular level the dendritic branching results in semi-globular to globular structures, mostly with a high density of functionalities on the surface together with a small molecular 'volume'. The higher

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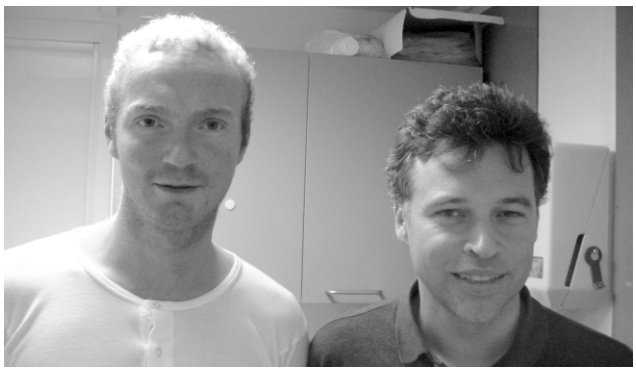

Ulrik Boas (left) and Peter Heegaard (right)

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generation dendrimers occupy a smaller hydrodynamic volume compared to the corresponding linear polymers, due to their globular structure. However, in comparison with globular proteins, the dendrimers have a bigger hydrodynamic volume.

The dendritic structure is characterised by 'layers' between each focal point (or cascade) called generations (shown as circles on Fig. 1). The exact numbering of generations has been the subject of some confusion (see *e.g.* reference 7). In this review, the dendrimer generation is defined as the number of focal points (cascade points) when going from the core to the surface, a generation 5 (G5) dendrimer thus has 5 cascade points between the core and the surface.

The core is sometimes denoted generation 'zero' (G0), as no cascade points are present. For a polypropylene imine (PPI) dendrimer, the core is 1,4-diaminobutane which has no cascade points, for a polyamido amine (PAMAM) 'Starburst™' dendrimer the core is ammonia *etc*. (hydrogen substituents are not considered a focal point). In PAMAM dendrimers the intermediate compounds having carboxylate surface groups are denoted half-generation dendrimers, that is dendrimers of *e.g.* G1.5 or G2.5.

The dendrimer design can be based on a large variety of linkages, such as polyamines (PPI dendrimers),[2] a mix of polyamides and amines (PAMAM dendrimers)[4] or built up by more hydrophobic poly(aryl ether) subunits.[8] More recent examples are dendrimer designs based on carbohydrate[9] or calixarene core structures,[10] or containing 'third period' elements like silicon or phosphorus,[11] just to give a few examples, Fig. 2. Due to the vast number of dendrimer designs and synthetic approaches used to create these impressive structures, the present review will have as the main focus, the biomedical uses of the commercially available PAMAM and PPI dendrimers and their interaction with biological systems.

1.1 A brief depiction and nomenclature of dendrimers

The 'full picture' of *e.g.* host–guest interactions involving dendrimers can become severely crowded and confusing. When considering surface-modified dendrimers and binding of guest molecules either to the surface or to the outer shell, a briefer depiction of the dendrimer can be applied. Instead of drawing the complete dendrimer structure, the inner shells are depicted as a black 'ball' (see Fig. 3); an italic number beneath the ball is the number of functional groups on the depicted surface. In this way the host–guest interactions become clearer. For a G5-PPI dendrimer this number will be 64, when only the terminal amino groups are depicted. The number of outer shell functionalities (pincers) in a G5-PPI dendrimer will be 32.

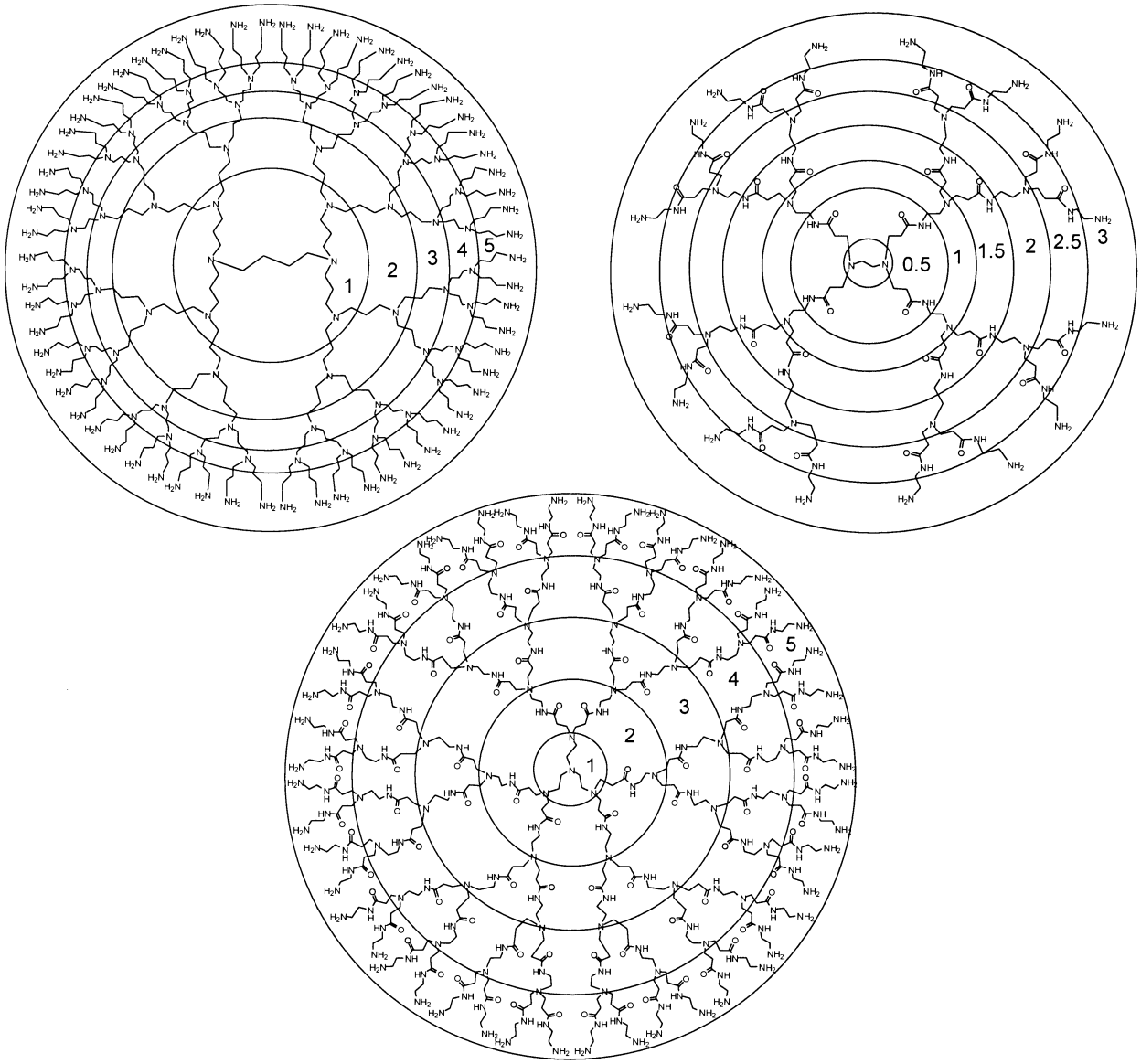

Fig. 1 Common commercially available dendrimers. Top left: Polypropylene imine dendrimer (G5). Top right: Polyamido amine dendrimer (G3). Bottom: Polyamido amine (Starburst™) dendrimer (G5). Each generation is marked with a circle.

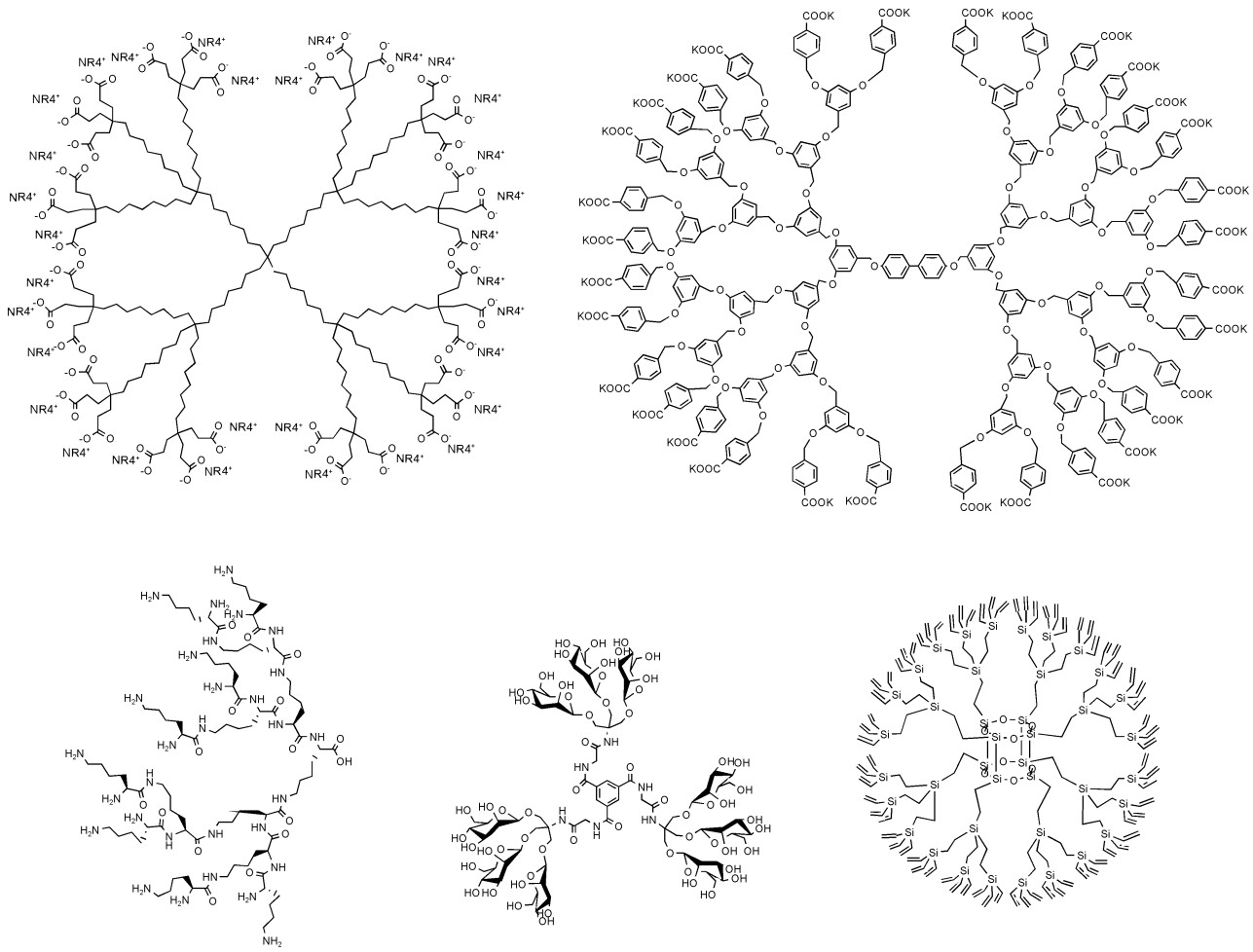

Fig. 2 Various designs of dendrimers. Top, left: Unimolecular micelle.[12] Top, right: Poly aryl ether dendrimer.[13] Bottom, left: Polylysine.[14] Bottom, middle: Carbohydrate dendrimer.[9] Bottom, right: Silicon based dendrimer.[15]

1.2 Chemical synthesis of dendrimers

Dendritic structures are chemically synthesised by two different approaches, either divergent or convergent, Fig. 4. In the divergent approach the dendrimer is synthesised from the core as the starting point and built up generation by generation. However, the high number of reactions which have to be performed on a single molecule (with a large number of equivalent reaction 'sites'), demands very effective transformations (99+% yield) to avoid defects. Even for very efficient transformations per generation the yield of 'perfect' G5-PPI dendrimer will only be approximately 25% by the divergent method.[22,23] The alternative convergent approach developed by Hawker and Frechét[13,24] starts from the surface and ends up at the core, where the dendrimer segments (dendrons) are coupled together. In the convergent approach, only a small number of reactive sites are functionalised in each step, giving a small number of possible side-reactions (or 'missing' reactions) per step. Each synthesised generation of dendrimer can therefore be purified, although purification in the higher-generation dendrons becomes more cumbersome, because of increasing similarity between reactants and formed product. However, with proper purification after each step, dendrimers without defects can be obtained by the convergent approach, Fig. 4.

By the convergent method it is also possible to create intriguing asymmetric dendrimeric structures for example by joining two different dendronic segments together in a controlled fashion.[25]

1.3 The dendrimer structure and the intrinsic properties of its compartments

As the dendritic structure grows, several compartments arise. The dendrimer structure can be divided into three parts:

–The multivalent surface, with a high number of potential reactive sites.

–The 'outer shell' just beneath the surface having a well-defined microenvironment protected from the outside by the dendrimer surface.

–The core, which in higher generation dendrimers is protected from the surroundings, creating a microenvironment surrounded by the dendritic branches.[26]

The interior is thus well-suited for encapsulation of guest molecules. The three parts of the dendrimer can be tailored specifically for the desired purposes, *e.g.* as dendritic sensors, drug vehicles or drugs. The multivalent surfaces on a higher-generation dendrimer can contain a very high number of functional groups. This makes the dendritic surfaces and outer shell well-suited to host–guest interactions where the close proximity of a large number of species is important.

1.4 Physicochemical properties of dendrimers

Already early in the history of dendrimers it was suggested that the 3-dimensional nanosized structure of the higher generation dendrimers would make this class of synthetic molecules suitable as mimics of proteins.[27] It must be kept in mind, however, that in contrast to proteins which consist of folded, linear polypeptide chains, the branched architecture of the dendrimer interior is to a large extent formed by covalent bonds, resulting in a somewhat less flexible structure. In addition, the dendrimer is on average less compact than a protein, *i.e.* interior is not packed as efficiently as in typical proteins, and the dendrimer contains a substantially higher number of surface functional groups than proteins of comparable molecular weight, Table 1).

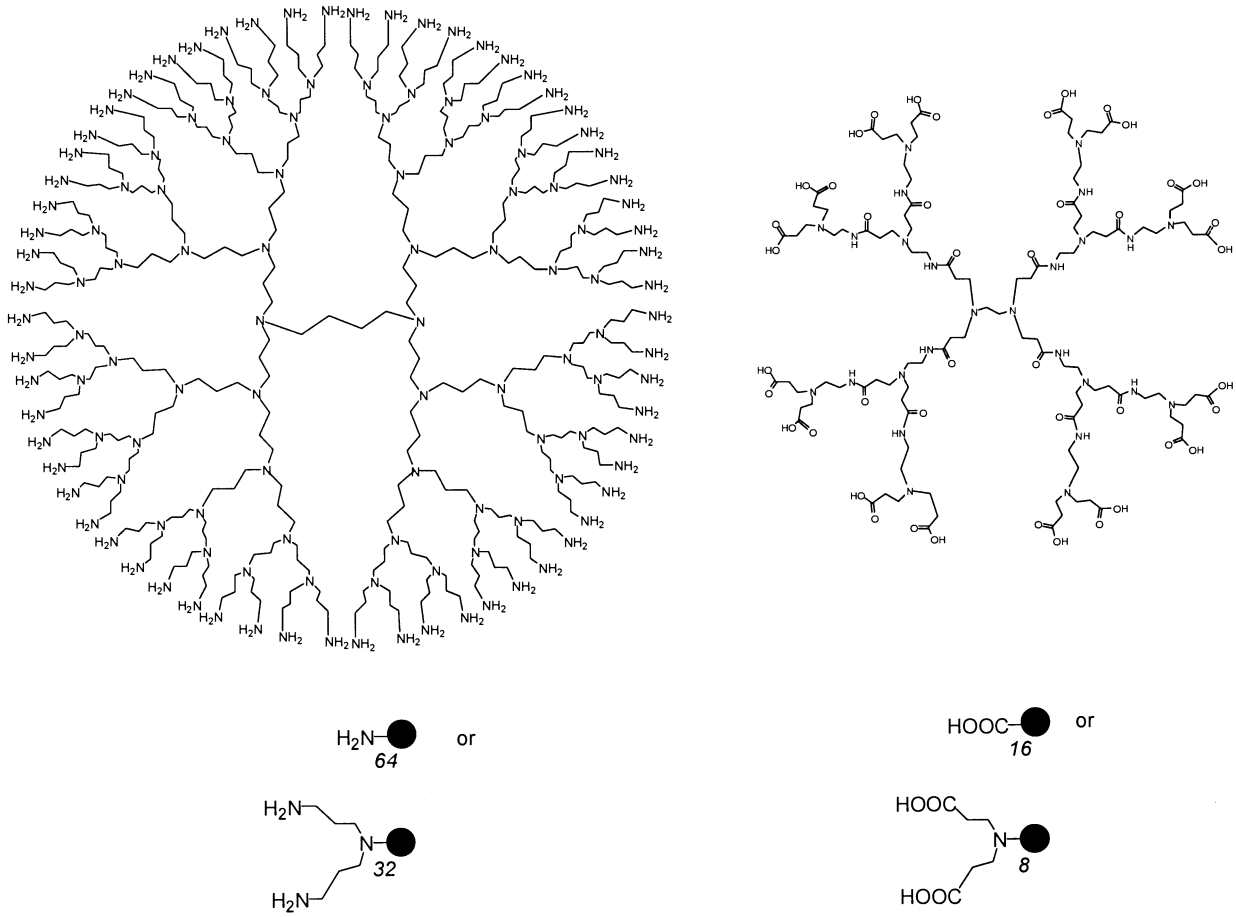

Fig. 3 'Black ball' depiction of, left: G5-PPI dendrimer; right: G2.5-PAMAM dendrimer.

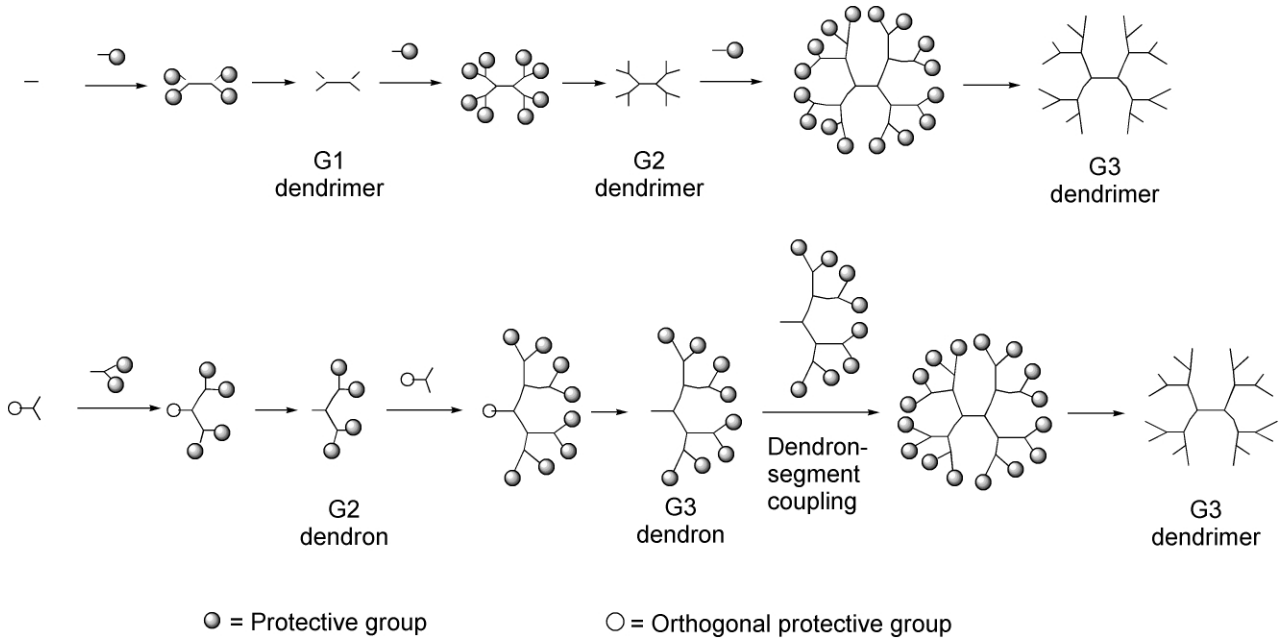

Fig. 4 Dendrimer synthesis (schematically depicted). Top: Divergent strategy. Bottom: Convergent strategy

Molecular dynamic studies carried out by several research groups on dendrimers show that the dendrimers, similar to proteins, can adapt 'native' (*e.g.* more tight) or 'denaturated' (*e.g.* extended) conformations dependent on the polarity, ionic strength and pH of the solvent. Amino-terminated PPI and PAMAM dendrimers (that is dendrimers having primary amines as surface groups) exhibit extended conformations upon lowering of pH because electrostatic repulsion between the protonated tertiary amines in the interior as well as between the primary amines at the dendrimer surface, forces the dendrimer branches apart[28] (Fig. 5). At pH >9 back-folding occurs as a consequence of hydrogen bonding between the interior protonated tertiary amines and the primary surface amines, resulting in a denser core.[29] The pH-related conformational changes are dependent on the nature of the charged group at the dendrimer surface. For PPI dendrimers having surface carboxylic groups 'small angle neutron scattering' (SANS) and NMR

Table 1 Comparison between dendrimers and biological entities. Selected physicochemical parameters

Type of molecule	Molecular weight	pI/surface charge	Diameter	Number and type of surface functional groups[a]
G3-PAMAM (Starburst[b])	2411	/+	2.2 nm[16]	12 primary amines
G6-PAMAM[c]	28 788	11/+[17]	6.5 nm[18]	128 primary amines
G6-PAMAM-OH	28 913	9/0[17]	–	128 hydroxyls
Medium sized protein (ovalbumin)	43 000	5/+ and −	5 nm[21]	20 primary amines 10 phenol groups 4 thiols, 7 imidazoles[19]
Large protein (Keyhole Limpet Hemocyanin)	~5 000 000	/+ and −	—	approx. 2000 primary amines, 700 thiols, 1900 phenols[20]
Virus[21]	~40 000 000	—	50–200 nm	—
Prokaryotic bacteria[21]	—	mainly negative	1–2 μm (30 nm cell membrane and cell wall)	—
Eukaryotic cell[21]	—	mainly negative	20 μm (9 nm cell membrane)	—

[a] protein functional groups not necessarily surface localised [b] core group is trifunctional, branches are made up of tris(aminoethyl)amine, methyl acrylate and ethylenediamine building blocks; Starburst is a Trademark of Dendritech Inc., Midland, MI, US. [c] Core group is tetrafunctional, branches are made up of methyl acrylate and ethylenediamine building blocks.

measurements of the diffusion coefficients in aqueous buffer, show that these dendrimers have the most extended conformations at pH 4 and pH 11, Fig. 5. This may be due to electrostatic repulsion between the protonated cationic inner tertiary amines at low pH, and electrostatic repulsion between the negatively charged deprotonated carboxylates at the dendrimer surface at high pH forcing the dendritic branches apart.

At pH 6 the carboxy-terminated PPI dendrimer has no net charge, resulting in a tighter conformation controlled by intramolecular hydrogen bonding.[30] Molecular density measurements at this pH show a homogeneous molecular density over the whole dendrimer, indicating a substantial degree of back-folding *i.e.* hydrogen bonding between terminal groups and groups in the core region, Fig. 6.

The polarity of the solvent greatly influences the 3-dimensional structure of dendrimers, and has been subject for conflicting views and discussions. Initial theoretical studies by de Gennes and Hervet on unmodified PAMAM (Starburst™) dendrimers using a self-consistent mean-field model, concluded that in good solvents (that is solvents with a high ability to solvate the dendritic structure), the dendrimers had the highest molecular density at the periphery, leading to dense packing of the surface groups upon increasing generation.[31] Calculations by Lescanet and Muthukumar proposed a uniform molecular density throughout the dendrimer, indicative of a pronounced degree of backfolding.[32] Later, calculations by Murat and Crest concluded, contrary to de Gennes, that the highest molecular density in PAMAM dendrimers was located near the core, independent of the solvent conditions.[33]

Recent NMR studies performed on PPI dendrimers indicate that an apolar solvent such as benzene will favour polar intramolecular interactions (*e.g.* hydrogen bonding) resulting in back-folding of the dendrimer arms into the dendrimer interior, whereas the increased acidity of chloroform, will increase solvation of the dendrimeric structure *via* hydrogen bond donation to the interior tertiary amines resulting in a more extended conformation of the dendrimer.[34] Both theoretical as well as experimental studies on amino functionalised PPI and PAMAM dendrimers reflect the tendency of an apolar solvent (poor solvent) to induce a higher molecular density in the core region due to back-folding (intramolecular polar interactions), and lower molecular density at the surface. In polar solvents the dendrimer arms are solvated and the molecular density at the dendrimer surface is increased.[35]

NMR and SANS studies performed by De Schryver's group on aryl ether dendrimers with a rubicene core, show that these dendrimers have extended conformations in a π-interacting solvent such as toluene, whereas a polar solvent such as acetonitrile induces a conformational collapse, probably due to strong intramolecular π-interactions.[36] In surface modified PPI dendrimers capable of hydrogen bonding between surface functionalities (end groups), the intramolecular hydrogen bonding tends to be enhanced upon increasing generation of the dendrimeric system, as a consequence of closer vicinity of the surface functionalities in the higher generation dendrimers.[37–39] Furthermore, experimental studies show that the hydrogen bonding end groups are located at the periphery of the dendrimer, supporting de Gennes' dense shell packing model.[40]

A microenvironment can arise in the dendrimer core as a consequence of limited diffusion of solvent molecules into the dendrimer. As an example, dendrimers dissolved in polar solvents such as aqueous media can have a very apolar interior (unimolecular micelle) allowing organic molecules to be encapsulated and carried in aqueous media. As we shall see, this property of dendrimers makes this class of molecules very well-suited as carriers of various bioactive substances.

2 Properties of dendrimers in biological systems

2.1 The significance of multivalency in biological interactions

Multivalent interactions can be found throughout nature, ranging from the divalent binding of antibodies and many biological receptors to the multimillion-valent interactions of a Gecko's foot-hair.[1] Multivalency has been shown to lead to a strongly increased activity compared to the corresponding monomeric interaction. This synergistic enhancement of a certain activity *e.g.* catalytic activity or binding affinity from a monomeric to a multimeric system, is generally referred to as the 'cluster'- or 'dendritic' effect.[9,41–43] The dendritic effect is attributed to a co-operative effect in a multivalent system leading to a larger increase in activity than expected from the valency of the system (*i.e.* additive increase). It is thus important to differentiate between different phenomena:

–with a higher number of binding entities per molecule in a dendrimer substance there is a simple increase in the mole to mole efficiency of binding (one mole of ligand corresponds to several moles of binding entities), *i.e.* an additive effect;

–a dendritic effect (or cluster-effect) comes into play when the simultaneous attachment to *n* binding entities in the same ligand molecule leads to an synergistic increase in affinity with a maximum binding affinity of (single ligand affinity)^n. The cluster effect has especially been observed for carbohydrate–protein receptors in natural systems with the glycoside cluster effect as a classic example.[44]

It is important to realise that multivalency can also increase the specificity of a given interaction,[41] essentially by increasing both the (high) affinity for the specific ligand and the (low) affinity for

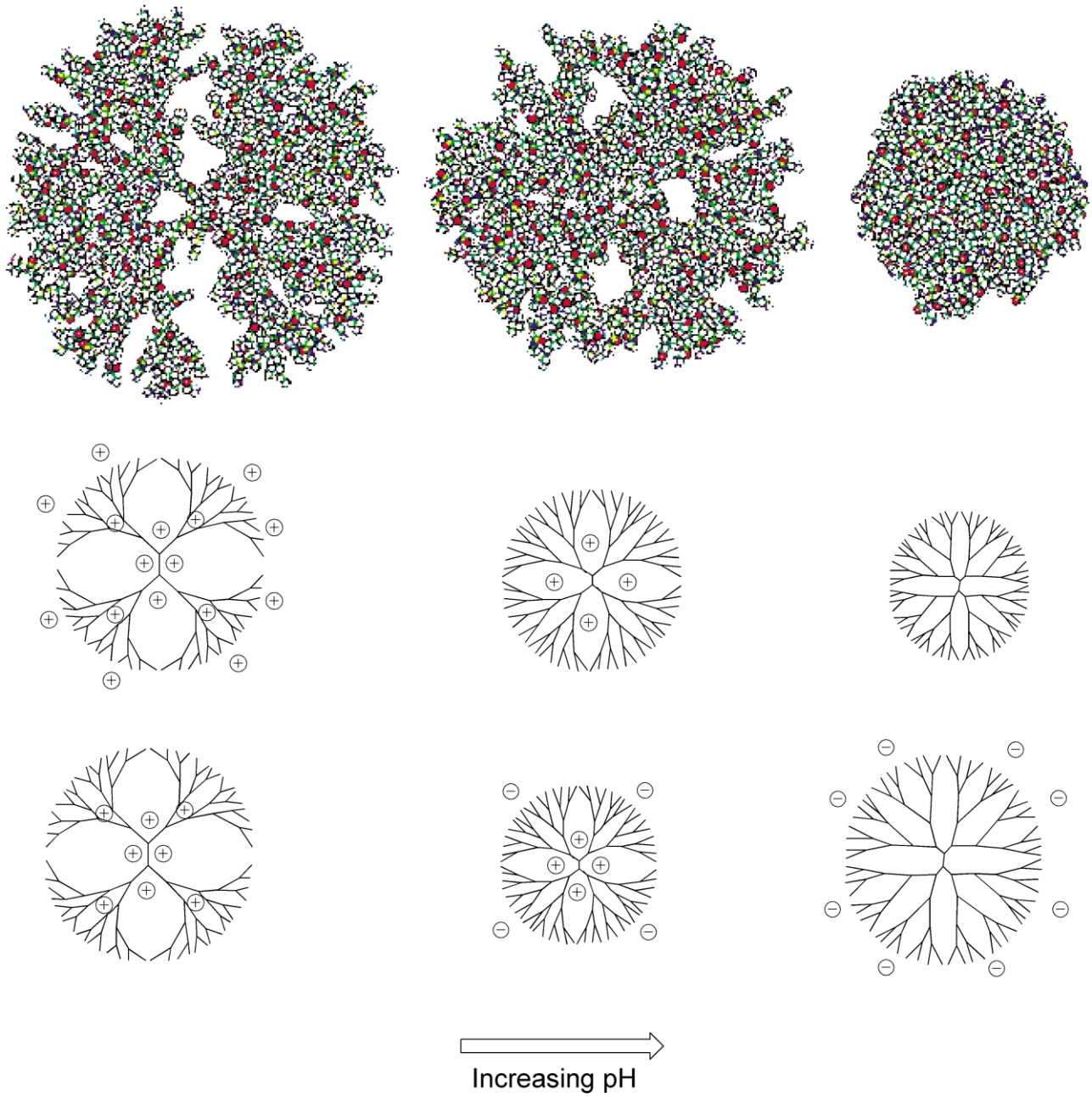

Fig. 5 Top row: Three dimensional depiction of conformational change of an amino-terminated PAMAM dendrimer at increasing pH (reprinted with kind permission from reference 28, copyright (2002) American Chemical Society). Middle row: Two-dimensional depiction of the conformational change of an amino-terminated PAMAM dendrimer upon increasing pH. Bottom row: Two-dimensional depiction of the conformational change of a carboxy-terminated PPI dendrimer at increasing pH.[30]

the unspecific ligand geometrically, leading to a much higher ratio of the specific affinity compared to the unspecific affinity.

Biological systems are replete with examples of multivalent interactions[45] and this can be rationalised by the fact that multivalent interactions provide:

–tight binding from rather low-affinity binding of single ligands;

–a possibility of utilising low-affinity ligands in a new arrangement to cope with an evolutionary new binding partner;

–more efficient cell-cell interactions mediated by multiple interactions.

Factors that play a role in the binding of dendrimeric multivalent ligands include obviously the geometry of the multimerically presented ligands and the flexibility of their attachment to *e.g.* a dendrimer, Fig. 7.[46]

Dendrimers are perfectly suited to supply multivalency of synthetic or semisynthetic, biologically interesting entities in a spatially well-defined manner.

2.2 Biocompatibility of dendrimers

In order to apply dendrimers as tools for drug design or as drug delivery devices *in vivo*, they have to fulfil several biological demands of crucial importance. The dendrimers should be:

– non-toxic;

– non-immunogenic (if not required *e.g.* for vaccines);

– able to cross biobarriers such as, *e.g.* intestine, blood-tissue barriers, cell membranes *etc*.;

– able to stay in circulation for the time needed to have a clinical effect;

– able to target to specific structures.

2.2.1 *In vitro* toxicity. *Dendrimers with cationic surface groups*: Not only dendrimers but cationic macromolecules in general cause destabilisation of the cell membrane and result in cell lysis.[47] The exact mechanism of cytotoxicity caused by these polycationic structures has not yet been fully revealed.

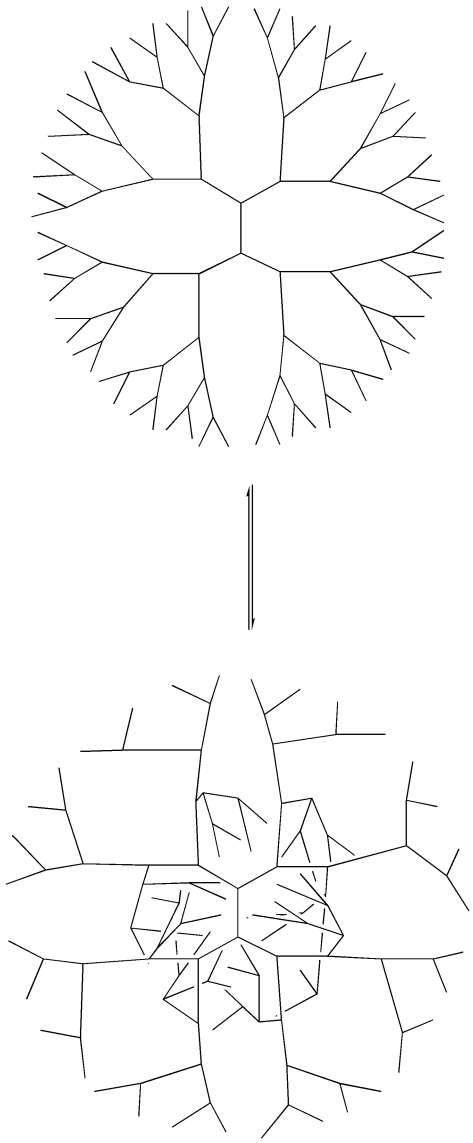

Fig. 6 Variation in molecular density of a dendrimer due to back-folding. Increased back-folding leads to an increased molecular density in the core region (bottom). The degree of back-folding varies with solvent polarity and pH.

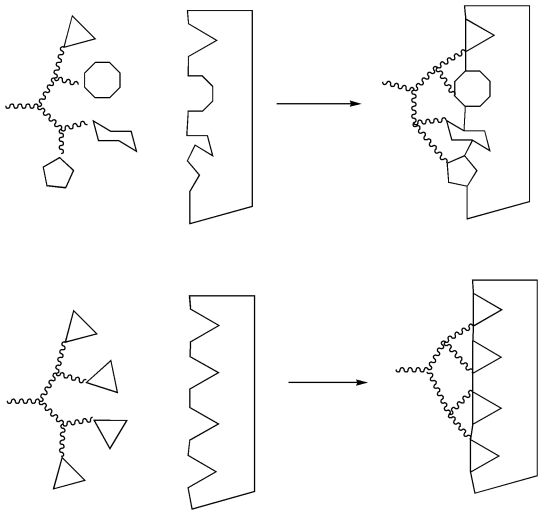

Fig. 7 Surface organisation of a hetero-functionalised dendrimer (top), or a homo-functionalised dendrimer (bottom), upon interaction with an appropriate receptor.

Initially, comparative toxicity studies on different cell-lines concluded that amino-terminated PAMAM (Starburst™) dendrimers had lower cytotoxicity than the lysine based dendrimer, polylysine 115 with LD_{50} = 25 μg mL^{-1} and LD_{50} > 300 μg mL^{-1} for polylysine 115 and G6-PAMAM, respectively.[48] However, later *in vitro* cytotoxicity by IC_{50} measurements (the concentration where 50% inhibition of mitochondrial dehydrogenase activity is measured) on amino-terminated PAMAM dendrimers showed a significant cytotoxicity of this class of compounds on human intestinal adenocarcinoma Caco-2 cells, albeit, with some variation in IC_{50} values.[49,50] In addition, the cytotoxicity has shown to be generation dependent for amino-terminated PAMAM dendrimers, with the higher generation dendrimers being the most cytotoxic.[16,49,51] This is in accordance with the general finding that increasing molecular size of polymers may result in increased cytotoxicity.[51] Also, haematotoxicity studies using amino-terminated PAMAM dendrimers conclude that the dendrimer has haemolytic effect on a solution of rat blood cells which increases with increasing dendrimer generation.[52] Myotoxicity studies on rodent muscles isolated from male Sprague Dawley rats showed that the amino-terminated G4-PAMAM dendrimer was more myotoxic than cationic liposomes and proteins.[53] Other studies on neuroblastoma cells in culture incubating with up to 7.4 μg mL^{-1} of PEI, PPI and PAMAM dendrimers for 1 week showed cytotoxicity of the amino-terminated PAMAM and PPI dendrimers.[7]

Recent studies have shown that amino-terminated PAMAM dendrimers, with their globular and less flexible structures, have lower toxicity than more flexible amino functionalised linear polymers. This can be explained by the lower adherence of the less flexible and globular dendrimeric structures to cellular surfaces. The degree of substitution on the amine functionality has found to be important as well, with primary amines being more toxic than secondary or tertiary amines.[51,54] This may be explained by the increased shielding of the positive charge from nitrogen by the larger molecular size of *e.g.* alkyl substituents compared to the hydrogen atoms found in a primary amine.

For amino-terminated PPI dendrimers a similar generation dependent increase in cytotoxicity has been found, with the higher generation dendrimers being most cytotoxic (IC_{50} < 5 μg mL^{-1} for G5-PPI).[55] As with the PAMAM dendrimers, the PPI dendrimers showed a generation dependent haemolytic effect on blood cells, with the high generation dendrimers being most haemolytic.[52]

In summary, amino-terminated dendrimers are generally cytotoxic.[52] The cytotoxicity of the cationic dendrimers can be explained by the favoured interactions between negatively charged cell membranes and the positively charged dendrimer surface, enabling these dendrimers to adhere to and damage the cell membrane, causing cell lysis.

Dendrimers with anionic surface groups: Recent comparative toxicity studies of anionic and cationic amino-terminated PAMAM dendrimers using Caco-2 cells, similarly conclude that the amino-terminated PAMAM dendrimers have a significantly higher cytotoxicity compared to the anionic carboxyl functionalised 'half-generation' PAMAM dendrimers.[49] Lower generation PAMAM dendrimers having anionic (*e.g.* carboxylate) surface groups, show neither haematotoxicity nor cytotoxicity at concentration of 2 mg mL^{-1}.[52] However, the biocompatability of dendrimers is not solely determined by the nature of their surface groups. Dendrimers based on an aromatic polyether skeleton having anionic carboxylate groups on the dendrimer surface have been shown to be haemolytic on 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.[52]

Surface derivatisation and cytotoxicity: Upon partial derivatisation of the PAMAM dendrimer surface amines with chemically inert functionalities like PEG or fatty acids the cytotoxicity towards Caco-2 cells is reduced significantly (from IC_{50} ~ 0.13 mM to > 1 mM). This can be explained by reduction of the overall positive

charge when transforming the basic primary surface amino groups to non-charged amides as well as encapsulating the dendrimer cationic interior tertiary amines. It was found that partial derivatisation with lipid or PEG (6 lipid chains and 4 PEG chains on a G4-PAMAM, respectively) lowered the cytotoxicity. However, upon introduction of a larger number of lipid- or PEG-chains no reduction was observed and a high number of lipid chains increased the cytotoxicity in this system, probably due to cell lysis by hydrophobic interactions.[49]

Additives and cytotoxicity: Additives can lead to a significant reduction in toxicity of amino-terminated dendrimers, where for example the addition of fetal calf serum together with PAMAM dendrimers, partially modified with the fluorophore Oregon Green, reduced the cytotoxicity towards human carcinoma (HeLa) cells in comparison with the partially modified dendrimer alone.[56] In these systems the toxicity was further reduced by complexation with oligonucleotides. Studies performed on PAMAM dendrimers aiming for gene delivery, similarly conclude that DNA complexed amino-terminated PAMAM dendrimers of low generation (up to G3) do not possess any significant cytotoxicity *in vitro*, and that the toxicity of the dendrimers is reduced upon complexation to DNA.[57,58]

Unmodified, amino-terminated PPI dendrimers are similarly less cytotoxic when formulated with DNA for transfection, with the lower generation dendrimers (G2) being the best transfection agents.[55] This could indicate that the non-covalent binding between the dendrimer and protein or DNA lead to a shielding effect of the polycation, similar to what is obtained by covalent modification of the dendrimer surface amines.

However, these observations are contradicted by other cytotoxicity studies on polycation–DNA complexes.[59] These studies show the same or higher cytotoxicity when unmodified amino-terminated G5-PAMAM dendrimer was formulated with DNA; albeit, the higher toxicity of the DNA–dendrimer complexes is not directly attributed to toxicity of the cationic amino-terminated dendrimer, but rather to the cellular stress upon transfection with high levels of DNA (3 μg mL^{-1}), which may lead to apoptosis.[60] Furthermore, Gebhart and coworkers suggest that the amino-terminated G5-PAMAM dendrimers still have positively charged amines on the surface of the dendrimer–DNA complex due to their rigidity, which could also retain the cytotoxicity of the complex.

2.2.2 *In vivo* toxicity. Only a few systematic studies on the *in vivo* toxicity of dendrimers have been carried out so far. The general observation is that injections into animals (mice) with 10 mg kg^{-1} concentrations of PAMAM dendrimers (up to G5) do not appear to be toxic, independent of whether they are unmodified or modified at the dendrimer surface.[16,61] Furthermore, it has been found that injection of unmodified amino-terminated PAMAM dendrimers together with ovalbumin in mice did not result in any significant toxicity *in vivo* (no weight loss, no granuloma formation, no haemolysis or inflammation), but that these mixtures had adjuvant activity.[62]

Recently, new hydroxy- or methoxy-terminated dendrimers based on a polyester scaffold (Fig. 8) have shown to be non-toxic both *in vitro* and *in vivo*.[63,64] At very high concentrations (40 mg mL^{-1}) these polyester dendrimers induced some inhibition of cell growth *in vitro* but no increase in cell death was observed and, upon

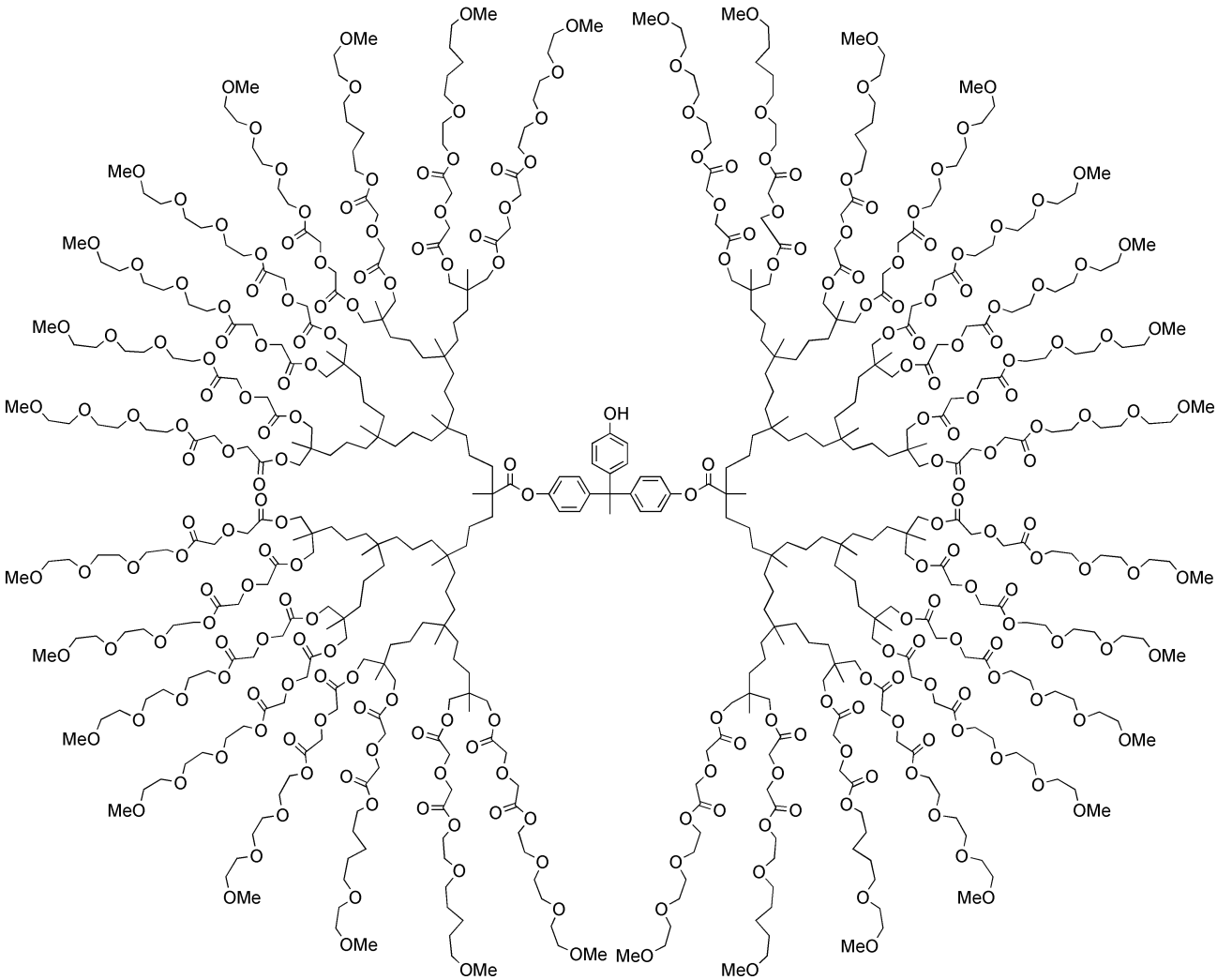

Fig. 8 Non-toxic polyester based dendrimer (G4) well-suited for delivery of drugs.[63,64]

injection in mice, no acute or long-term toxicity was observed. The non-toxic properties make these new dendritic motifs very promising as biodegradable drug delivery devices, as the dendrimer may be degraded by hydrolytic enzymes after the release of the drug.

2.2.3 Immunogenicity. Initial systematic studies performed on unmodified amino-terminated PAMAM dendrimers showed no or only weak immunogenicity of the G3–G7 dendrimers.[16,62] However, later studies showed some immunogenicity of these dendrimers and found that modification of amino-terminated PAMAM dendrimers with polyethylene glycol (PEG) chains reduces immunogenicity and gives longer lifetime in the blood stream in comparison to unmodified dendrimers.[65] The PEG chains increase the hydrophilicity of the dendrimer, and create a highly hydrated dendrimer surface with low disturbing effect on the physiological environment. However, as we shall see, the dendrimer surface can alternatively be modified with antigens or T-cell helper epitopes creating highly immunogenic compounds.

2.2.4 Biopermeability. As mentioned, dendrimers complexed to DNA can be transported into the cell nucleus, with less membrane damage and fewer cytotoxic effects compared to free dendrimer. Several approaches have been investigated to further increase the transfection ability of the dendrimer–DNA adducts, and new dendrimer motifs continue to be developed for that purpose.[58,66] *In vitro* transfection studies concluded that addition of moderate amounts of sulfonated β-cyclodextrins (β-CD's) enhances the transfection ability of the PAMAM dendrimer–DNA complexes, due to ionic binding between the negatively charged sulfonate moieties of the β-CD and the cationic amino-terminated dendrimer, leading to an altered DNA–dendrimer complex composition.[67] In polylysine dendrimers, the transfection efficacy was enhanced by derivatisation of the dendrimer surface with PEG; however, the overall level of transfection was low, presumably due to low release of the DNA from the dendrimer.[68] The investigations generally conclude that the spherical shape of dendrimers is not an advantage in gene delivery, which agrees with earlier work, where 'fragmented' PAMAM dendrimers show superior transfection efficacy in comparison with the spherical 'complete' dendrimers.[69]

In order to use dendrimers for drugs or drug delivery, their biopermeability on a macroscopic level also has to be taken into consideration. *In vivo* studies on the ability of cationic amino-terminated PAMAM dendrimers (G1–G4) to cross the microvascular endothelium indicate that the extravasation time across the microvascular endothelium increases with increasing generation and molecular weight of the dendrimer.[70] Studies on the transepithelial transport of PAMAM dendrimers (G0–G4) in Madin–Darby canine kidney cells showed that the G4-PAMAM dendrimer possessed the largest permeability; however, in these studies no linear dependence between the dendrimer generation and permeability was found.[71] Para-cellular transepithelial transport of amino-terminated PAMAM dendrimers in a Caco-2 cell monolayer showed higher permeability for the lower generation dendrimers (G0–G2) in comparison with the higher generation dendrimers, which were also hampered by their increasing cytotoxicity.[50] *In vitro* studies on anionic PAMAM dendrimers ('half-generation' PAMAM dendrimers) on an everted rat intestinal sac system showed that these dendrimers rapidly crossed into the intestine of adult rats. The transfer rate of the dendrimers was faster than other polymeric systems, suggesting that these dendrimers could be useful as building blocks in oral delivery systems.[72] Polylysine dendrimers where the surface has been modified with lipid chains and studies of their uptake through the intestine of rats concluded that these lipid modified dendrimers had poorer uptake in comparison with well-known delivery systems such as polystyrene latex particles.[73]

3 Development of host–guest binding motifs in dendrimers towards biological applications

In the previous section, we considered dendrimer toxicity in biological environments, and in some cases it was found that dendrimers can become less toxic upon interaction with additives such as *e.g.* DNA; in these cases structurally undefined complexes between the dendrimer and the DNA are formed. In this section we will take a closer look at different motifs which are useful for complexation between dendrimers and various guest molecules, the so-called host–guest complexes, and their possible use in drug research. The use of dendrimers as hosts or carriers of smaller guest molecules is a research area of increasing interest and has been extensively reviewed.[74–76]

3.1 Dendrimers as hosts

PAMAM and PPI dendrimers, with their large molecular size and multivalent surfaces, serve as good scaffolds for synthetic macromolecular hosts, and subsequent surface modification can yield a host molecule with the desired properties. The host–guest binding can either take place in the cavities of the dendrimer core ('endo-receptor'), or at the multivalent surface or outer shell of the dendrimer ('exo-receptor').[74] One early example of an endo-receptor is the 'dendritic box',[77–81] where a G5-PPI dendrimer was modified at the surface with Boc-protected phenylalanine. In this way the outer shell was made more dense due to the sterically demanding Boc-protective groups. Guest molecules of different size, present during the modification of the dendrimer, were encapsulated in the interior and isolated from the bulk by the densely packed Boc-phenylalanine surface. The dendrimer could simultaneously bind up to 4 large guest molecules (Rose Bengal) and 8–10 small guest molecules (*p*-nitrobenzoic acid). Upon selective acidolysis (formic acid) of the Boc-groups at the surface, the surface shell became more open and the small guest molecules were allowed to leak from the dendrimer, whereas the large guest molecules remained trapped in the core, Fig. 9.

The large guest molecules could subsequently be released from the dendrimer by acidolysis of the amide bonds creating the unmodified dendrimer with a more open surface structure. In the dendritic box, the interactions between the host and the guest molecule were not tailored to be specific, but more governed by the molecular size of the guest molecule, and the physical size of the cavities in the host. By incorporating a biodegradable linkage in the dendrimer outer shell, the outer shell of the dendrimer host could alternatively be perforated by physiological or enzymatic hydrolysis, directing this host motif towards drug delivery applications.

3.1.1 Guest binding to the dendrimer core *via* hydrophopic interactions. The unimolecular micelle reported by Fréchet's group is based on a polyaryl ether dendrimeric network having carboxylate surface groups, and is capable of dissolving apolar guest molecules such as pyrene in water (Fig. 2). The amount of dendrimer was proportional to the amount of dissolved pyrene. The host–guest binding is assumed to be mediated through π–π interactions between the electron-rich aryl ether and the aromatic guest. This was confirmed by the enhanced ability to bind electron-deficient aromatic guests (π-interaction stabilised) and the decrease in binding of an electron-rich guest molecule (π-interaction destabilised due to electron repulsion) compared to pyrene.[8] These types of dendrimers would make good candidates for carrying hydrophobic bioactive compounds, *e.g.* steroids.

Dendrimers specifically tailored to bind hydrophobic guests to the core have been created by the Diederich group under the name 'dendrophanes'. The water soluble dendrophanes are centered around a 'cyclophane' core, and can bind aromatic compounds, presumably *via* π–π interactions, Fig. 10. These dendritic structures were shown to be excellent carriers of steroids.[82,83]

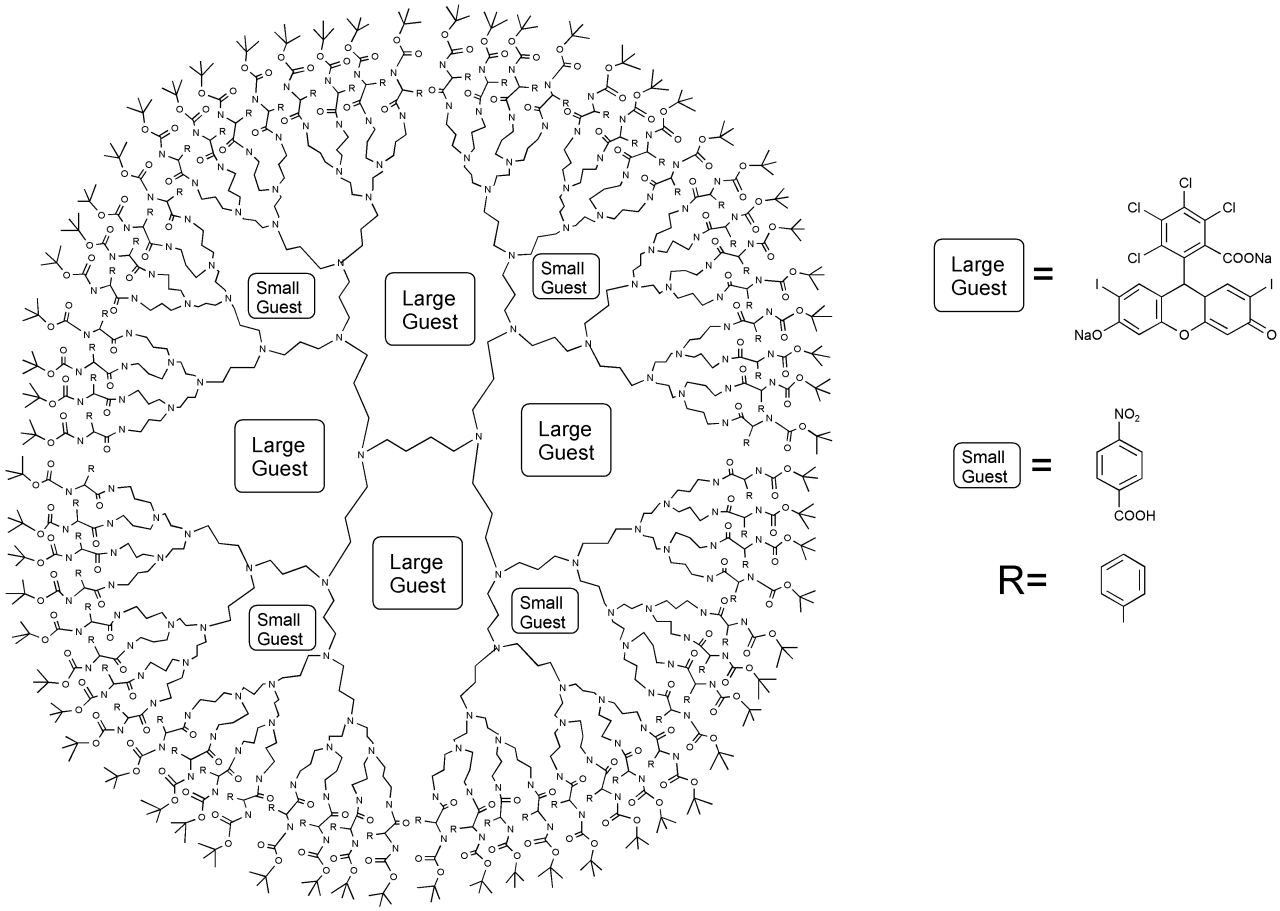

Fig. 9 The 'dendritic box'.[77–81]

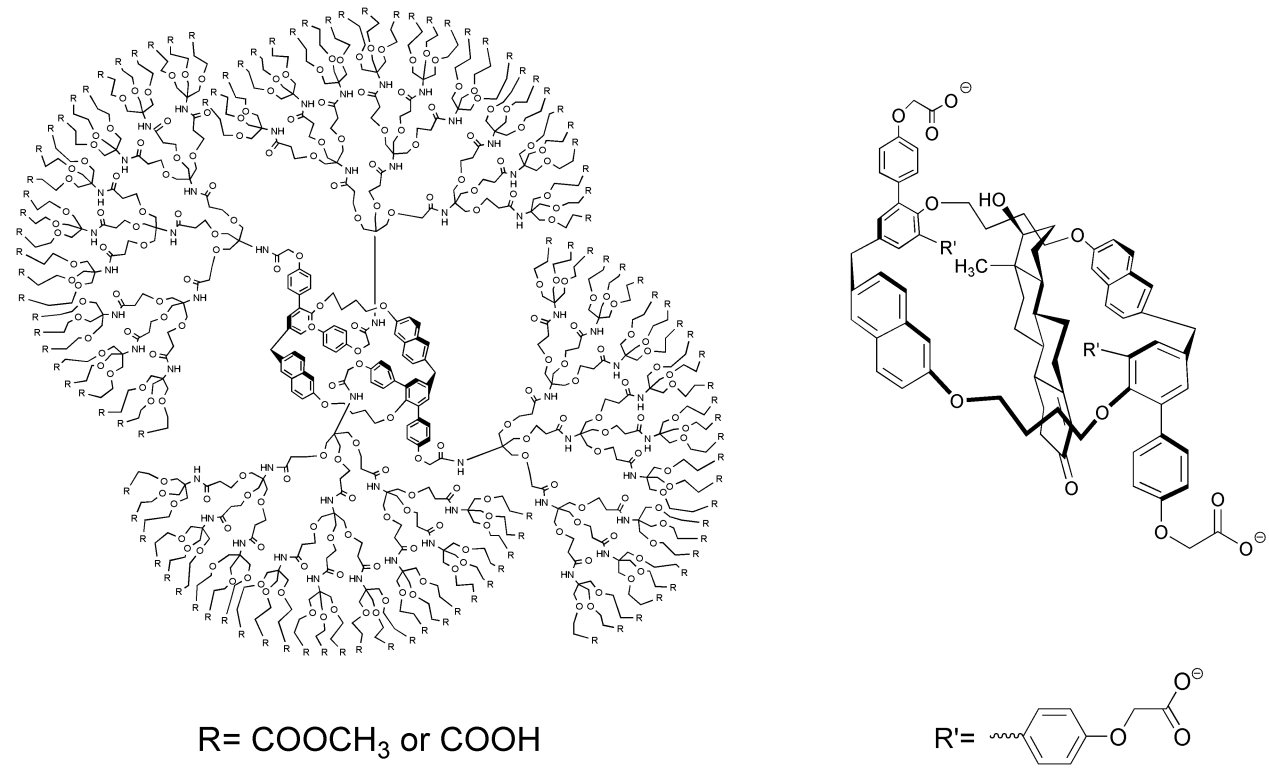

Fig. 10 Left: G3-Dendrophane for the encapsulation of steroids. Right: The host–guest binding motif upon complexation with testostorone.[83]

3.1.2 Guest binding to the dendrimer core *via* polar interactions. In order to be able to bind more polar bioactive compounds to the core of a dendrimer, Diederich and coworkers designed the so-called 'dendroclefts'.[84,85] These water-soluble dendrimers were centered around an optically active 9,9′-spirobi[9*H*-fluorene] core and showed a marked diastereoselectivity towards recognition of octyl β-D-glucoside over octyl α-D-glucoside, Fig. 11. Proton NMR analysis performed on the host–

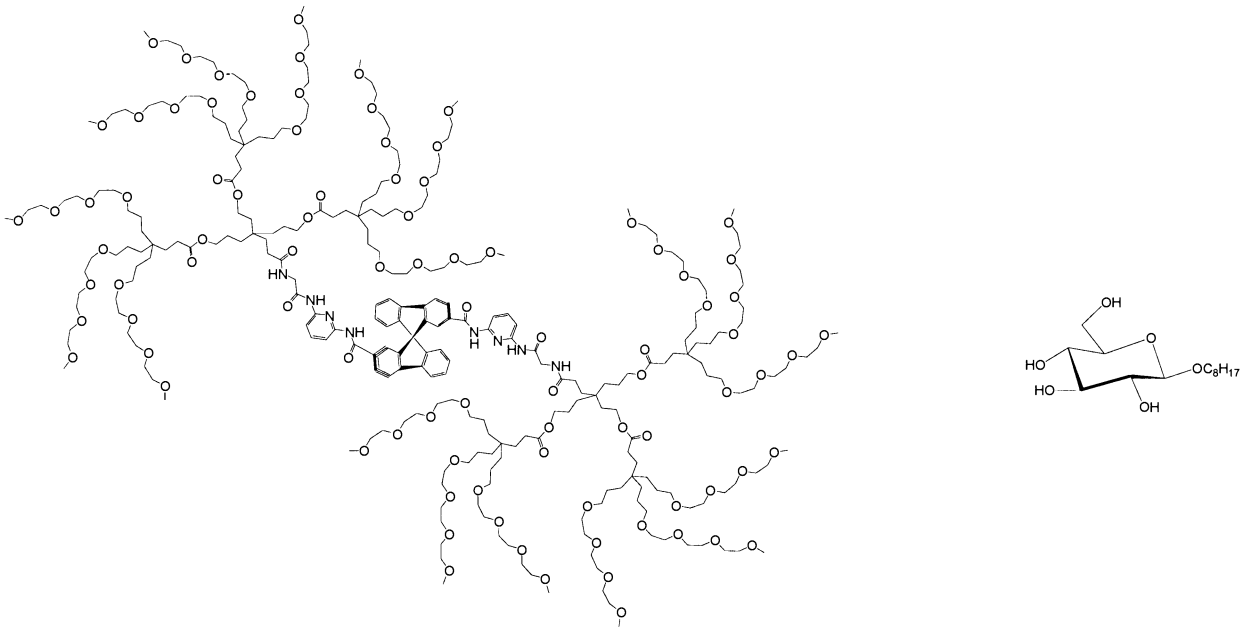

Fig. 11 G2-Dendrocleft (left), which can act as a diastereoselective host for octyl-β-D-glucoside (right) over the α-anomer.[84,85]

guest complexes showed that hydrogen bonding between the pyridine carboxamide moieties in the core and the oxygen atoms in the carbohydrate guest was the major contribution to the host–guest interactions.

The diastereoselectivity was found to increase with increasing dendrimer generation, probably due to increased hydrogen bonding between the bound carbohydrate guest and the alkyl ether oxygen atoms of the dendritic wedges.[84]

A simpler approach has been developed by Michell and coworkers, who modified PAMAM dendrimer surface amines with a glycerol derivative (tris(hydroxymethyl)aminomethane), thereby creating water-soluble dendrimers capable of binding acidic aromatic antibacterial compounds, which could be released by lowering the pH. The host–guest complex formation occurred *via* acid–base interactions and hydrogen bonding between the dendrimer inner tertiary amines and the acidic substrate, however, the exact nature of the host–guest interactions could not be determined by ^{1}H-NMR[86]

3.1.3 Dendrimers as temporary scaffolds. An interesting alternative approach to create hosts with highly defined dendritic structures has recently been developed by Zimmerman and coworkers.[87] Here the dendrimers can be used as scaffolds for preorganised structures in creating artificial hosts by 'molecular imprinting' inside a polymeric network of dendrimers. In the specific example, a dendrimer consisting of a porphyrin core and a surface containing terminal double bonds was used as 'monomer' and polymerised by Grubb's catalyst into a polydendritic network. Subsequently, the base labile ester bonds between the core and the dendritic wedges were cleaved, releasing the core porphyrin structure from the preorganised dendritic polymer (Fig. 12). In this way, a polymer containing 'porphyrin-shaped cavities' was obtained and it was shown that this polymer was capable of binding porphyrins with association constants of 1.4×10^5 M^{-1}.[87] This 'poly-dendrimer' can be regarded as a synthetic porphyrin-recognising antibody.

3.2 Binding to the outer shell of dendrimers – the 'click in' concept

The 'multi-pincer' structure of the PPI dendrimers opens up intriguing possibilities for binding various guest molecules to the outer-shell pincers of a surface modified dendrimer. One example is the urea modified PPI dendrimers used for binding of oxo-anions to the outer shell urea groups *via* hydrogen bonding, as shown by Vögtle and coworkers.[88] Meijer's group used urea- and thiourea-functionalised PPI dendrimers for binding guest molecules containing a urea–glycine 'tail' unit.[38,39] The guest molecules interact with the dendritic host by multiple urea (guest)–(thio) urea (host) hydrogen bonds and ionic interactions between the glycine carboxylic acid and the dendrimer outer shell tertiary amino groups. The concept was baptised the 'click in' mechanism, and it has been suggested that the acid–base reaction between the dendrimer and guest with subsequent Coulomb-attractions pulls the guest into the dendrimer, whereas the hydrogen bonding keeps the guest bound to the host. By intake of urea guests the outer shell becomes increasingly crowded and dense, hence this host–guest motif could provide a non-covalent example of a dendritic box (Fig. 13).

As the urea glycine tail is highly similar to the *C*-terminus of a peptide, it was investigated whether the dendrimer could act as a host or carrier for peptides, directing the 'click in' motif towards biological applications. This host–guest motif can be useful as a pH sensitive drug delivery system, where the peptide guest can be released upon lowering pH, as a result of protonation of the carboxylic acid moiety. It was found that the urea and thiourea modified dendrimers were capable of binding different peptides, regardless of the bulkiness of the side chains, and that the peptides could be released from the dendrimer under mild acidic conditions.[89,90] As the dendrimer binds different peptides without selectivity, this would introduce the possibility of using the dendrimer as host ('bus') for several different peptides ('passengers') simultaneously, Fig. 14.

4 Dendrimers as drug delivery devices

The research in dendrimer mediated drug delivery has mainly been focused on the delivery of DNA drugs (genes or gene inhibitors) into the cell nucleus for gene or anti-sense therapy, and numerous reports have been published on the possible use of unmodified amino-terminated PAMAM or PPI dendrimers as non-viral gene transfer agents, enhancing the transfection of DNA into the cell nucleus.[57,58,91–95] The exact structure of these host–guest binding motifs has not been determined in detail, but is presumably based on acid–base interactions between the anionic phosphate moieties in the DNA backbone and the primary and tertiary amines in the dendrimer, which are positively charged under physiological conditions. It has been found that partially degraded (or fragmented) dendrimers are better suited for gene delivery than the

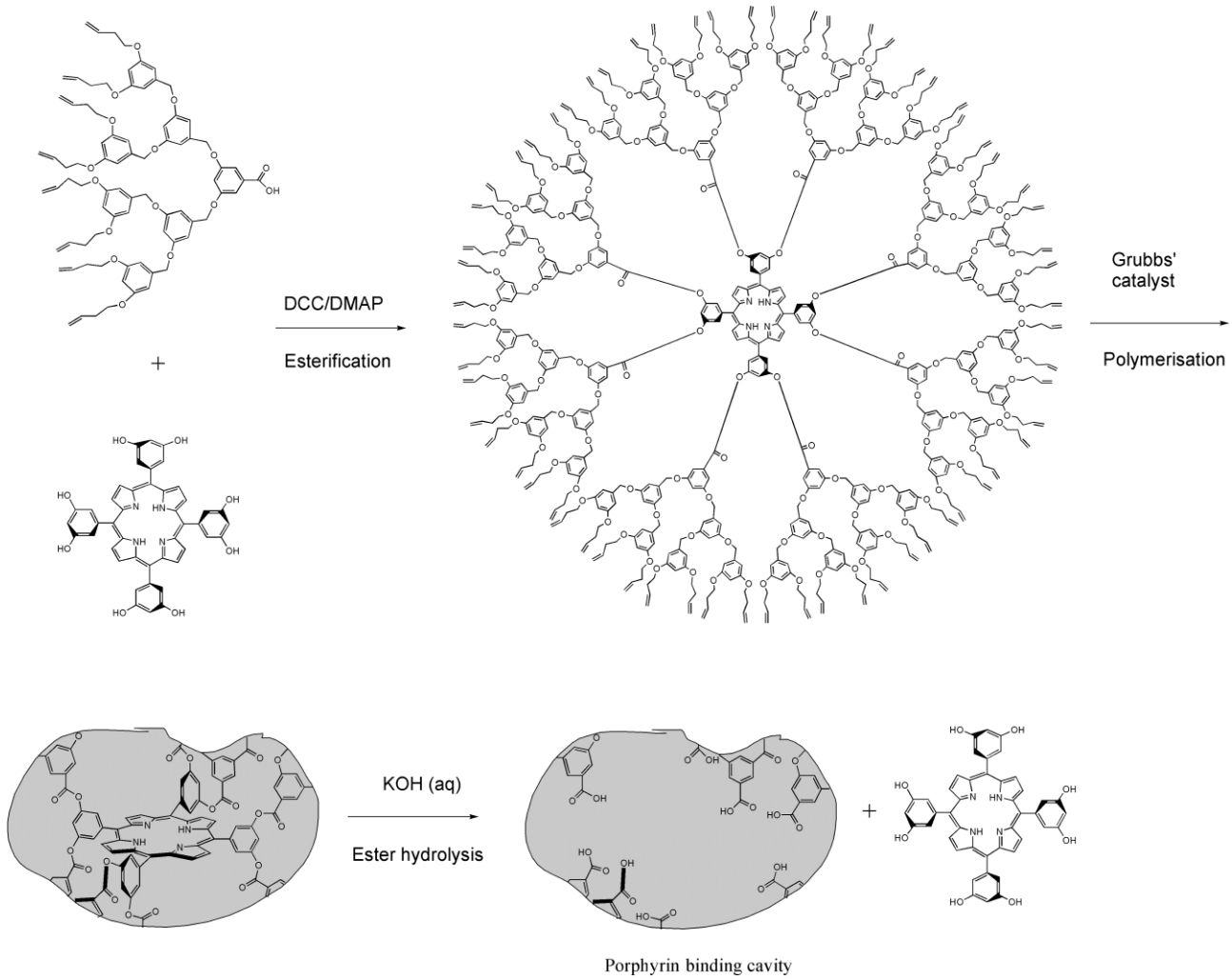

Fig. 12 The use of porphyrin core dendrimers as scaffolds in creation of porphyrin binding hosts by molecular imprinting.[87]

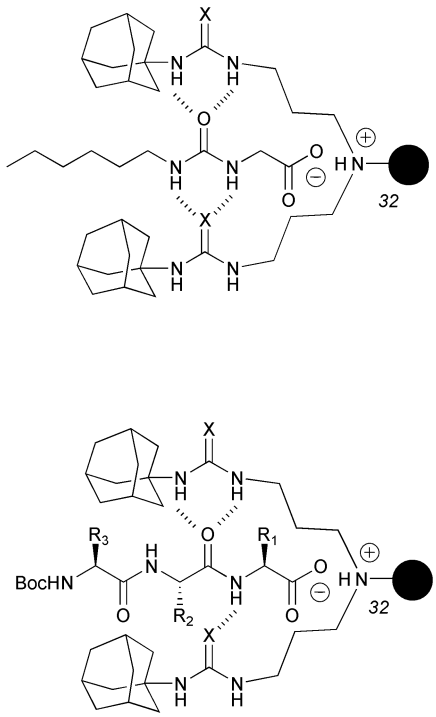

Fig. 13 The 'click-in' design. X = O,S. Top: Intake of urea containing guest molecules in urea or thiourea hosts.[38,39] Bottom: Intake of *N*-Boc-protected peptides.[89,90]

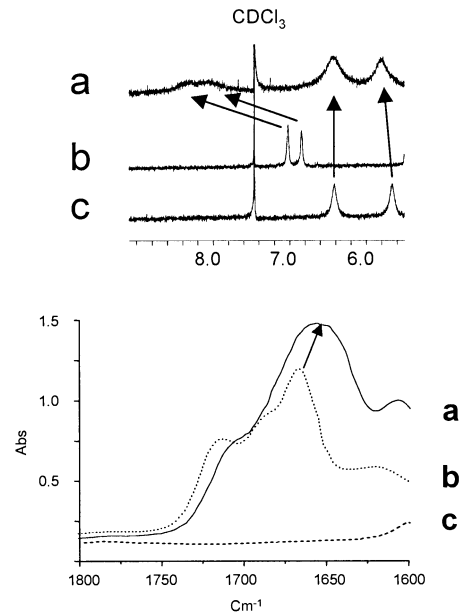

Fig. 14 Investigation of host–guest hydrogen bonding interactions in 'click-in' between a peptide guest and a thiourea modified dendrimer in $CDCl_3$ by ^{1}H-NMR (top) and IR (bottom), a) Dendrimer–peptide complex, b) uncomplexed peptide and c) uncomplexed dendrimer. The increased hydrogen bonding upon complex formation results in a downfield shift in NMR and a shift towards lower wavenumbers in IR.[89,90]

complete dendrimers (*vide supra*), and a fragmentation (activation) step consisting of hydrolytic cleavage of the amide bonds is needed to enhance the transfection efficiency, Fig. 15.[69,96,97]

In comparison to the intact dendrimers, the partially degraded dendrimers have a more flexible structure (fewer amide bonds) and form a more compact complex with DNA, which is preferable for gene delivery by the endocytotic pathway.[97] In addition, it is generally found that the maximum transfection efficiency is obtained with an excess of primary amines to DNA phosphates, yielding a positive net charge of the complexes. The more flexible higher generation PPI dendrimers (containing no amides) are found to be too cytotoxic for use as non-viral gene vectors, however, the lower generations are well-suited for gene delivery.[55]

The unmodified amino-terminated dendrimers transport the DNA to the cell membrane and may help in the transfection process by disruption of the cell membrane. The transfection of free DNA will be hampered by electrostatic repulsion between the negatively charged phosphate groups in the DNA backbone and the negatively charged cellular membrane, Fig. 16.

Cationic, amino-terminated dendrimers which are partially covalently modified with drugs, can be useful as extracellular 'stickers' in 'extracellular matrix-targeted local drug delivery,' giving a very high local concentration of the carried substrate or drug close to the cellular surface.[98] However, this drug (gene) delivery technique is only appropriate if a particular drug or gene has to be introduced into a broad range of cells. In order to obtain a specific cellular treatment, drug vehicles that direct the drug only to specific cell types can be designed. One example of such cell-specific dendritic drug vehicles is a dendrimer derivatised with folic acid (pteroyl-L-glutamic acid). Folic acid is an important substrate for uptake in cells by the *folate receptor pathway*. As the folate receptor is over-expressed in cancer cells, these folic acid derivatised dendrimers are taken up by cancer cells preferentially to normal cells, making these dendrimers well-suited for the cancer-specific drug delivery of cytotoxic substances.[99,100] Very recently, folate modified PAMAM dendrimers have been successfully used as carriers of boron isotopes (^{10}B) in boron neutron-capture treatment of cancer tumors.[101] An earlier report concluded that the use of unmodified amino-terminated PAMAM (Starburst™) dendrimers as boron carriers in combination with monoclonal antibodies had low *in vivo* tumor localising properties and gave rise to accumulation in the liver.[102,103] PAMAM dendrimers conjugated to the well-known anticancer drug *cis*-platin act as macromolecular carriers for platinum. The dendrimer-platinate gives a slower release of the platin, and shows higher accumulation in solid tumors and lower toxicity compared to *cis*-platin.[104] PAMAM dendrimer–

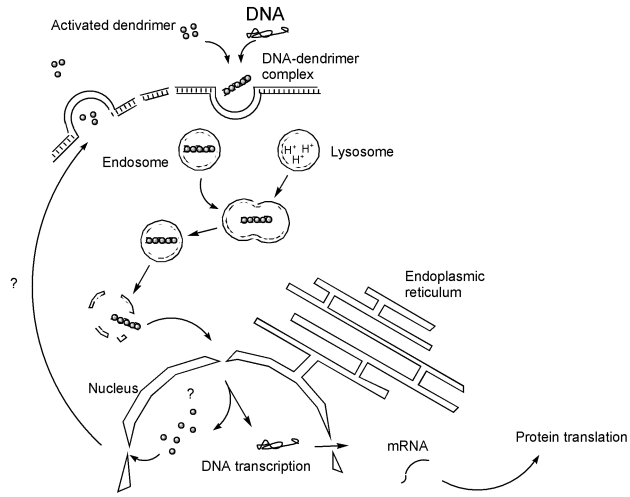

Fig. 16 Proposed scheme for the transfection of DNA into the cell nucleus aided by 'activated' dendrimers.

silver complexes that slowly release silver have shown anti-microbial activity against various Gram positive bacteria.[105]

The above mentioned approaches to drug delivery are based on non-covalent complex formation between the drug and the dendrimeric drug carrier. Another approach is to bind the drug covalently to the multivalent dendritic surface, *via* a biodegradable bond. Dendrimers based on a 1,4,7,10-tetraazacyclododecane core having primary amines at its surface have been partially modified with 1-bromoacetyl-5-fluorouracil to form a labile imide linkage. Upon hydrolysis of the imide under physiological conditions, the potent antitumor agent 5-fluoro-uracil could be released *in vitro*.[106]

4.1 Dendrimers as glycocarriers

Carbohydrates constitute an important class of biological recognition molecules. They differ from polypeptide recognition molecules in displaying a wider variety of spatial structures (due to possibilities of branching and anomericity) with fewer building blocks resulting in a high specificity combined with quite low binding affinity constants, often in the 10^{-5}–10^{-6} M range.[107] Carbohydrate binding proteins are called lectins[108] and lectin–carbohydrate interactions have been described in numerous cases in the immune system (cellular activation events), in bacterial and viral infections, in relation to cancer and cell growth *etc*. Carbohydrate-based drugs are therefore of interest as microbial

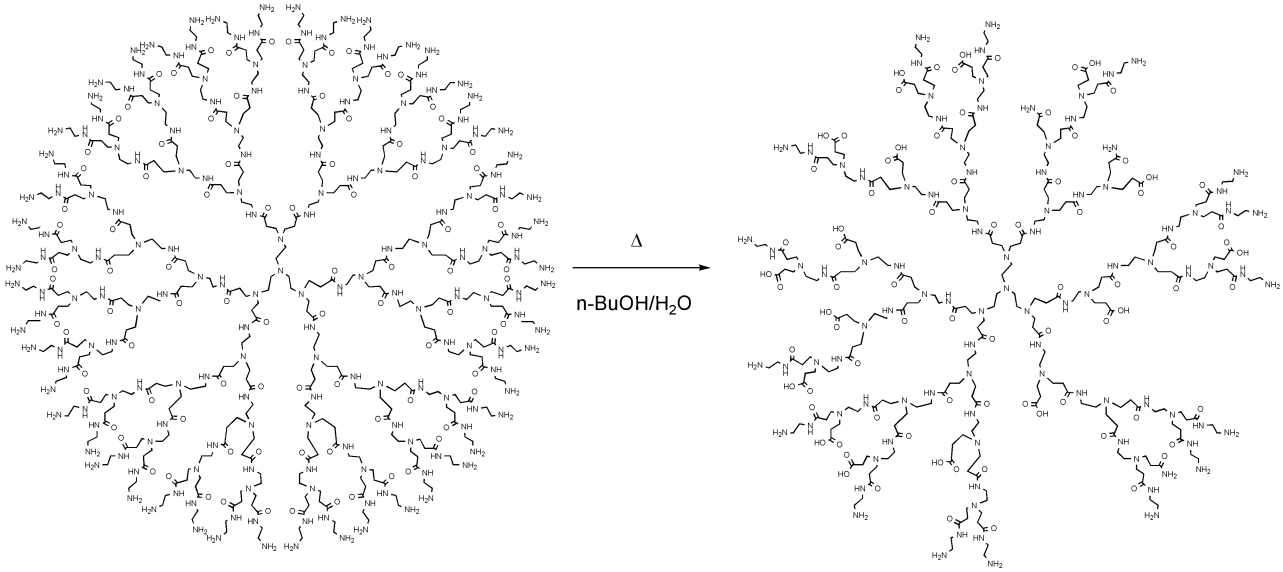

Fig. 15 'Activation' of a G5-PAMAM (Starburst) dendrimer by solvolysis, leading to partial hydrolysis of amides in the dendrimer.

anti-adhesins, microbial toxin antagonists, anti-inflammation drugs, antiviral and anticancer drugs. However, there are synthetic difficulties in obtaining such bioactive carbohydrate ligands. A strategy to overcome this utilises the multivalency/cluster effect (*vide supra*) obtainable by dendrimer presentation, to create carbohydrate ligands with adequate binding affinities from simple mono- or oligosaccharides. In biologically relevant structures, multivalent interactions between relatively short saccharides and lectins/carbohydrate receptors, (which as a rule contain multiple, identical carbohydrate binding sites) have also been observed and made possible by multivalent presentation of saccharide binding determinants, thus taking advantage of the cluster effect. This is seen for example in glycoproteins with multiple, identical "antennae" emanating as branches from a central attachment point. Some of these so-called "glycans" in fact resemble dendrimers very much, even down to the geometrical increase in terminal saccharide units from "G0" to "G3", Fig. 17[107]. Multivalency may also be achieved by lateral rearrangement of individual glycoconjugates, *e.g.* glycolipids in cell membranes. An estimate of the distances between the terminal monosaccharide units in such glycans has been attempted[109] for oligomannoside triantennary glycopeptide structures which mimic mammalian high-mannose glycans. The maximum distance attainable between the oligosaccharide branches was found to be below 30 Å (3 nm).

A recent review of carbohydrate receptors in nature with an emphasis on multivalency and the importance of the cluster effect is found in a review by Bezouska,[110] and several reviews concerning the biomedical use, design and synthesis of glycodendrimers with a thorough review of a number of neoglycoconjugate types have been published.[9,111–113]

Generally, low-valency neoglycoconjugates (up to 16 saccharide units) give highly enhanced affinities while high-valency neoglycoconjugates, although having enhanced binding affinities, may also lead to unwanted immunological reactions. Low-valency constructs are inherently easier to analyse and errors will more easily appear in high-valency constructs. For example, MALDI-TOF MS and NMR data on mannose-functionalised PAMAM dendrimers (G2–G7) shows that incorporation efficiency decreases with increasing generation (G5–G7 give non-stoichiometric incorporations) which is attributed to dendrimer defects rather than problems with non-quantitative incorporation yields. NMR does not identify these problems, probably because of the high degree of symmetry in the glycodendrimer.[114]

Especially useful, non-immunogenic glycoconjugates include peptide supported saccharides and dendrimer-supported saccharides (glycodendrimers).[111] Such adducts have been prepared from G3-PAMAM or multibranched lysine dendrimers, *e.g.* by coupling between mannose isothiocyanate (or sialic acid, lactose and 3′-sulfo Lewis^x saccharides) and the terminal amines of the dendrimer. Enhancement of affinity towards a macrophage/monocyte localised mannose-specific lectin was 300–400 times for the octameric adduct, compared to the monosaccharide (mannose).

Other very efficient glycoconjugate receptor binders are trivalent peptide–*N*-acetyl galactosamine and galactose adducts, which bind with very high affinity to the hepatic asialoglycoprotein receptor. These glycoconjugates present three identical monosaccharides bound through spacers to a peptidic backbone at evenly spaced attachment points.[115] The asialoglycoprotein receptor is located in the cell membrane of hepatocytes and is an example of a multimeric mammalian lectin.[116] This receptor was shown previously to be specific for galactose and galactosamine and has a clear preference for desialylated, multiantennary oligosaccharides of many serum glycoproteins, binding with the following preference: triantennary > biantennary > monoantennary.[117,118] This represents a natural example of the cluster effect, showing several-thousand fold affinity increase going from one to three-ligand clusters. As the asialoglycoprotein receptor is important for adherence to-, and tranfection through- the cell membrane, new transfection agents based on combinations of nucleotides and glycoside clusters have been designed to bind and eventually to be internalised by this receptor (*vide supra*).

Other types of glycosylated dendrimers, their synthesis and their applications as antigens for diagnostic, vaccine and biochemical have been extensively been reviewed elsewhere[119,120]

Using PAMAM dendrimers (G2–G7)[114] the different generations of mannose terminated dendrimers were compared with respect to relative binding activities towards the mannose-binding plant lectin concanavalin A (con A) in a hemagglutination inhibition assay. Con A has mannose-binding sites spaced by 6.8 nm on both sides of the tetrameric protein. It was found that 8- and 16-mer glycodendrimers did not show any increase in activity (per monosaccharide entity) compared to the monosaccharide (methylmannoside), which was not surprising as these small dendrimers do not span the two binding sites of con A and thus no clustering effect could be expected. Surprisingly, the 32-mer mannose PAMAM (G4) had a higher relative activity, indicating a clustering effect, even though theoretically, the carbohydrate units are not spaced far enough to ensure multivalency towards the receptor. With the higher generation dendrimers multivalent binding was clearly indicated, although in the higher generations, steric effects might play a role in decreasing the interaction strength.

Glycodendrimers with the cancer-associated T-antigen disaccharide (βGal 1–3 αGalNac) present in 2–6 copies were synthesized based on *N*,*N*′-bis(acrylamido)acetic acid cores, and tested for binding to the galactose-specific lectin peanut agglutinin and a mouse monoclonal antibody directed against the T-antigen, and a valency dependent increase in affinity from 10^6 M^{-1} (monomer) to 10^8 M^{-1} (tetramer) was demonstrated. Interestingly, the hexamer showed a substantially lower overall apparent affinity, indicating that steric effects participate in addition to the valency effect.[121] This type of construct has potential application in the detection of malignant tissues expressing T-antigen receptors, and, ultimately for targeting drugs to such altered tissue (one example being breast cancer carcinomas). Moreover, these glycodendrimers are non-immunogenic and show a superior level of molecular definition (monodispersity) compared to other types of glycopolymers. Similar constructs with similar properties but based on PAMAM (G1–G4) have also been reported.[122] The synthesis and use in breast cancer therapy of such T-antigen containing dendrimers has been recently reviewed.[123]

Mannosylphenyl-functionalised PAMAM dendrimers (G1–G4) and their interactions with lectins (con A and PSA), were compared to those of monosaccharides in an inhibition assay.[124] The mannosylated dendrimers were up to 400 times more inihibitory than methyl-mannose monosaccharide, indicating a strongly dendr-

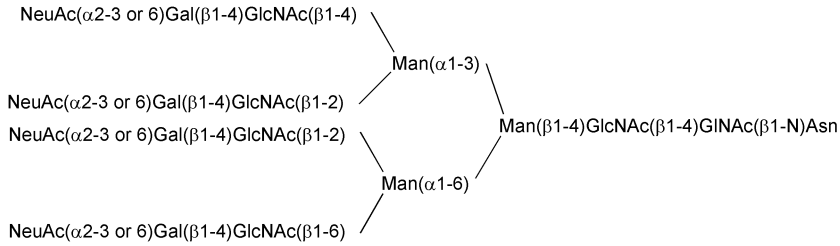

Fig. 17 Dendrimeric N-bound glycan (4-branched antenna).[107]

itic effect (a G4-PAMAM dendrimer carries 32 mannose surface units). However, no big increase in affinity was observed from the 8-mer (G2) to the 16 (G3) and 32-mers; most of the increase took place going from monomer to dimer and to the tetramer and 8-mer. The 8-mer generally showed the highest affinity increase per mannose residue (28 times increase for both lectins). In a very similar study, but emphasizing the significance of the precise spatial arrangement of identical disaccharide moieties on the binding to different types of sugar-binding biomolecules, André and coworkers[125] concluded that multivalent presentation of the carbohydrate units clearly increased the binding affinity towards plant lectins, while a lactoside-specific antibody bound less efficiently. Binding was also compared to binding of a conventional neoglycoprotein molecule containing the same carbohydrate constituents but having a more heterogenous presentation of the carbohydrate moieties. This was bound most efficiently by the antibody. These types of arylic PAMAM glycocarriers[124,125] adhere readily to plastic and are suitable for use as antigens in common solid-phase assays (*e.g.* ELISA).

In a structurally quite complicated version of the glycocarrier dendrimer theme Baussanne and coworkers[126] describe the synthesis of a decorated β-cyclodextrin, the β-cyclodextrin being conjugated in one position (at a primary hydroxyl group) with a dendritic "branch" (a dendritic "wedge") terminating in mannose residues; hereby a highly asymmetrical dendrimer is obtained which combines the hydrophobic drug carrier ability of β-cyclodextrin with the multivalent display of a biologically interesting monosaccharide. The use of a dendritic "branch" allows the attachment of multiple carbohydrate ligands to a single cyclodextrin molecule, without destroying the integrity and physicochemical properties of the cyclodextrin molecule, thus still allowing it to act as a hydrophobic drug carrier in aqueous solution. In this kind of construct, the dendritic branch can be said to function as a spacer separating a ligand cluster from a bioactive constituent (drug-carrier complex).

Reaction with the mannosyl-specific lectin concanavalin A was analysed by an inhibition solid- phase assay and yielded dissociation constants in the 10^{-4}–10^{-6} M range, increasing with the multimericity of the construct (from mono- to hexavalent), while the cyclodextrin moiety retained its ability to solubilise a hydrophobic drug. A fine analysability (NMR and mass spectrometry) was shown by 3,5-di(2-aminoethoxy)benzoic acid based dendrimers (G1–G3) decorated with lactose *via* a thiourea linkage.[127] Furthermore, there was a substantial increase in affinity for the lactose-specific (GM1 ganglioside specific) cholera toxin B-subunit (500 times increase for the octamer compared to monomer) in a soluble fluorescence assay. This might constitute an interesting route for the preparation of cholera therapeutics. These authors show, however, that the contribution to the affinity increase from the thiourea and phenyl groups is more than 70 times, almost by itself accounting for the total "synergetic effect" of the tetramer and half the "synergetic effect" of the octamer. In another study on bacterial carbohydrate-binding toxins (cholera toxin and *Escherichia coli* heat-labile toxin), the direct design of a pentavalent inhibitor glycodendrimer was described, based on the pentavalent binding of these types of toxins to cell surface glycolipids (GM1 gangliosides), and retaining the geometry of the natural ligands.[128] Also in this study the surface monosaccharides are combined with an aromatic substituent leading to an increased affinity. A high-affinity, non-aggregating interaction with the toxins was achieved and was characterised by X-ray diffraction and shown to be very similar to the structure of the toxin with its natural ligand. The scaffold used here was not a traditional dendrimer, but rather a coupling to a pentameric core-linker molecule.[128] Again, the importance of including an aromatic substituent in the ligand was shown as well as the exact geometry of the construct, especially the length of the "arms", and this could bring the affinity constant of the inhibitor into the range of the natural ligand – which is very important if such an inhibitor were to have any therapeutic use. The use of this type of toxin inhibitors is of some interest as there is no demand for them to cross the intestinal barrier and to enter the blood stream, as they are to block the interaction of the toxin with epithelial cells within the intestines.[128]

G1–G4 PAMAMs were used for the attachment of a modified disaccharide which was transformed into an active ester and then coupled to the surface amino groups, the disaccharide being the clinically important T-antigen (Gal β1–3 GalNAc), which is characteristic of certain cancers, in particular breast cancer carcinomas.[129] The idea was to use such constructs for interaction with carcinoma-related T-antigen binding receptors (thereby interfering with carcinoma growth) or to use them for generation of T-specific antibodies for diagnostic and therapeutic purposes. In an enzyme-linked immunoassay (ELISA) it was shown that the G4-adduct bound twice as much monoclonal antibody as the G3-adduct and four times as much as the G2-adduct. Interestingly, the G2-adduct (8-mer) was more than 25 times as efficient as the G1-adduct (tetramer), indicating a real dendritic effect going from G1 to the G2 PAMAM dendrimer, while no further dendritic enhancement was seen with G3 and G4 dendrimers. In a competition ELISA, 50% inhibition demanded approximately 500 times more monomer than G1-adduct (molar comparisons); on a per saccharide basis the inhibitory efficiencies of the dendritic structures were all similar and approx. 100 times the monomer. The discrepancy between the indirect and the blocking ELISAs goes uncommented (the tetramer being comparatively much less active in the indirect ELISA) but could be due to differences in coating abilities of the different dendrimer constructs which is also not discussed.

In conclusion, numerous studies have shown that glycosylated dendrimers are good mimics of natural glycoconjugates and will interact efficiently with natural carbohydrate receptors, in many cases to an extent that allows competition with natural binding substances.

5 Dendrimer drugs

5.1 Dendrimers as antiviral drugs

In general, antiviral dendrimers work as artificial mimics of the anionic cell surfaces, thus the dendrimers are generally designed having anionic surface groups such as sulfonate residues or sialic acid residues, which are acidic carbohydrates present at the mammalian cell surface. In other words, the dendritic drug competes with the cellular surface for binding of virus, leading to a lower cell-virus infection probability, see Fig. 18.

Polylysine dendrimers modified with naphtyl residues and having sulfonate surface groups have been found to be useful as viral inhibitors for Herpes Simplex virus *in vitro*.[61] The dendrimer works both as an inhibitor for virus entry and in late stages of virus replication.[130] PAMAM dendrimers covalently modified with naphthyl sulfonate residues at the surface, giving an polyanionic surface, also show antiviral activity against HIV. Also here the dendrimer drug works as an inhibitor for early stage virus/cell adsorption and at later stages of viral replication by interfering with the reverse transcriptase and/or integrase enzymes.[131,132] PAMAM dendrimers derivatised with sialic acid are efficient inhibitors of infection with influenza A subtype H3N2, however the inhibition is restricted to this influenza subtype.[133] A dendrimer with an amide surface has been designed and works as an inhibitor for the respiratory syncytial virus (RSV), see Fig. 19. The exact mechanism of action has not been revealed in detail, but may rely on hydrogen bonding interactions between the viral fusion protein and the dendrimer surface groups, causing inhibition of virus binding and fusion. However, even small alterations at the aromatic residues of the dendrimer decrease the antiviral activity and viral selectivity, suggesting that other binding modes *e.g.* π–π stacking could play a role as well.[134]

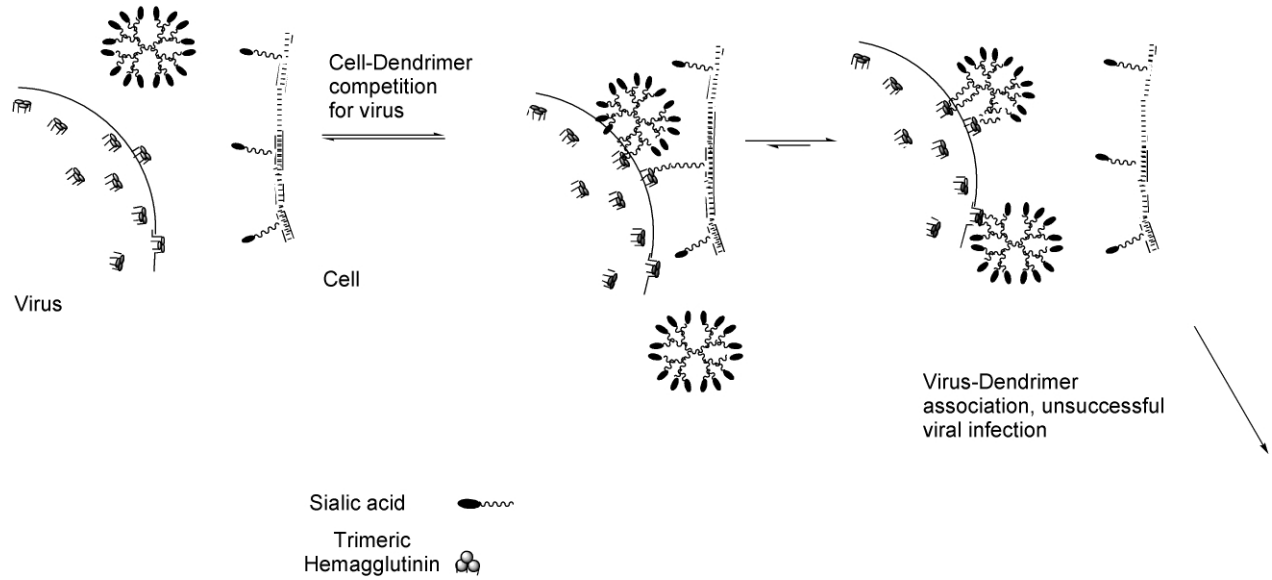

Fig. 18 Cartoon showing the anti-viral action of dendrimer drugs. Binding of the virus to the cell membrane, and subsequent penetration of the cell wall by the virus (endocytosis) is inhibited by the sialic acid decorated dendrimer drug.

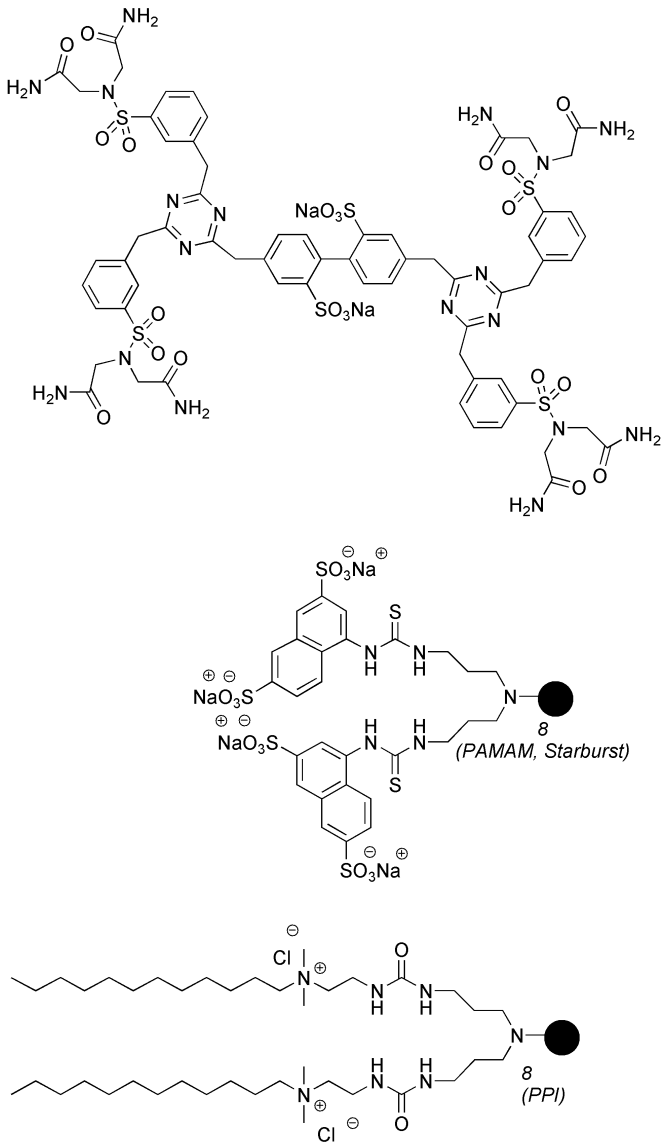

Fig. 19 Examples of molecular structures of anti-viral or anti-bacterial dendrimers. Top: Antiviral dendrimer, Respiratory Syncytial Virus.[134] Middle: Antiviral dendrimer, Herpes Simplex Virus.[61] Bottom: Anti-bacterial dendrimer, *E. coli*.[136]

5.2 Dendrimers as antibacterial drugs

In contrast to the antiviral dendrimers, the antibacterial dendrimers generally contain cationic surface functionalities such as amines or tetraalkyl ammonium groups (Fig. 19). The general mode of action of the antibacterial dendrimer is to adhere to and damage the anionic bacterial membrane, causing bacterial lysis. PPI dendrimers where the surface has been functionalised with tertiary alkyl ammonium groups have shown to be very potent antibacterial biocides against Gram positive and Gram negative bacteria.[135–137] The nature of the counter ion is important, as tetraalkylammonium bromides were found to be more potent antibacterials over the corresponding chlorides.[136] The dendritic biocides were found to have higher activity in comparison with other hyperbranched polymers. Polylysine dendrimers having mannosyl surface groups have been shown to inhibit adhesion of *E. coli* to horse blood cells in a haemagglutination assay, making these structures promising as antibacterial agents.[138]

5.3 Dendrimers as antitumor drugs

In photodynamic treatment (PDT) the drug becomes toxic upon irradiation by *in situ* formation of small amounts of singlet oxygen, which has strong physiologically damaging effects.[139] In comparison, the drug should be relatively non-toxic under non-irradiative conditions (low 'dark toxicity'), thus acting as a prodrug under non-irradiative conditions, see Fig. 20. Few reports have been published so far on the design of dendrimers containing various photosensitizers for the formation of singlet oxygen in the tumor tissue, but this research area may grow rapidly over the coming years. Dendrimers containing the photosensitizer 5-aminolevulinic acid at their periphery have been synthesised and are promising as agents for PDT of tumorigenic keratinocytes.[140] Polyaryl ether based dendrimers derivatised with the photosensitizer protoporphyrin, have been evaluated as candidates for the PDT of solid tumors.[141] The protoporphyrin derivatised dendrimers showed more specific cytotoxicity than protoporphyrin itself, and the dendrimers were more potent upon irradiation compared to protoporphyrin, probably due to an antenna effect of the dendritic wedges. The dendrimers showed a 140-fold lower dark-toxicity, compared to free protoporhyrin, a low dark toxicity is an important requirement in PDT, as high dark toxicity causes unspecific cytotoxicity.

6 Dendrimers as protein denaturants

Certain types of dendrimers act as chaotropes *i.e.* water structure perturbing solutes, lowering the dielectric constant and the

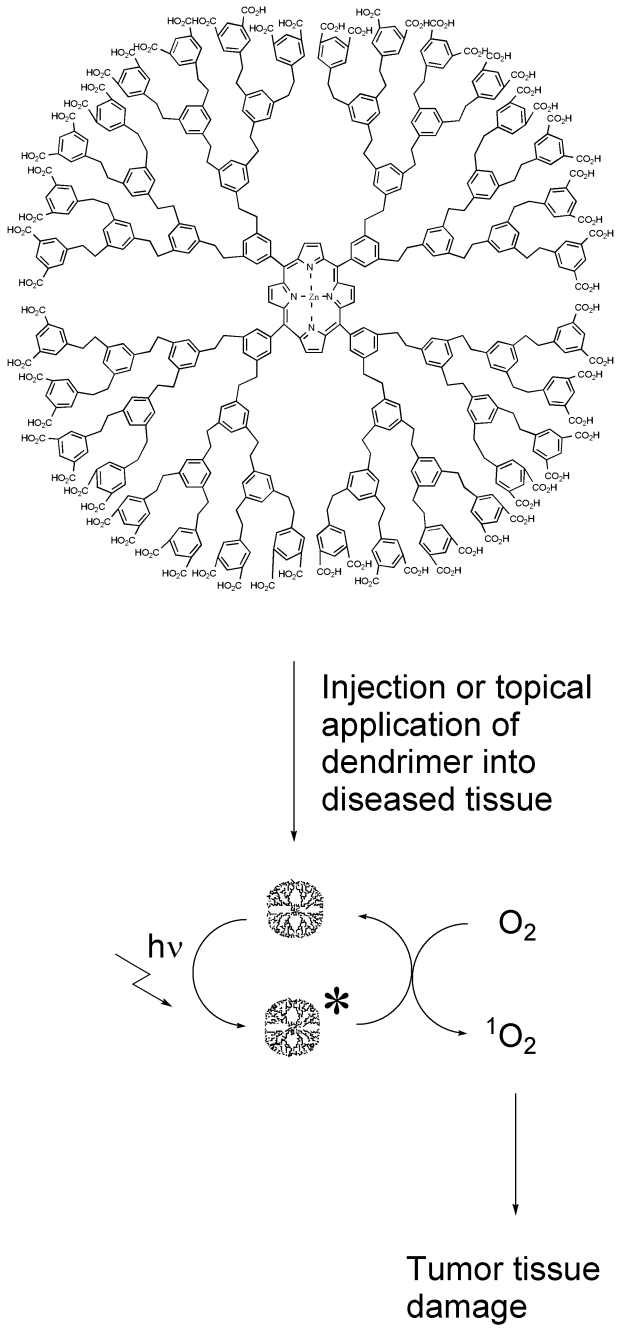

Fig. 20 Schematic depiction of 'photo dynamic therapy' (PDT) using a dendrimer with a protoporphyrin photosensitizer core, which upon irradiation with light and subsequent reaction with oxygen creates tissue damaging singlet oxygen.

viscosity of water; disordering the regular water structure by reorganising water molecules at the dendrimer surface. As with other chaotropes, this leads to hydrophobic interactions being disfavoured which, in turn, is highly destabilising for most protein tertiary structures (denaturation). Classical examples of chaotrophic salts are $MgCl_2$, urea, guanidinium chloride, sodium thiocyanate, guanidinium thiocyanate at high concentrations and other chaotropes include polarity-decreasing, water miscible organic solvents such as acetonitrile, propanol and methanol. Generally, chaotropes will serve to denature and solubilise proteins, which is useful for example in solubilising protein aggregates as are often encountered when expressing proteins in heterologous expression systems (inclusion body formation) and when extracting certain types of membrane proteins. Dendrimers, being compact, large polyionic substances have the physicochemical properties needed to make them potential chaotropes/protein denaturants,

A striking example of this was reported recently in the literature,[142] where cationic dendrimers were used for the solubilisation of prion protein aggregates. Prion proteins are able to attain a pathogenic structure/conformation in which they can cause mortal diseases called spongiform encephalopathies, including mad cow disease and Creutzfeldt-Jakob's disease. These deadly conformers are characterised by their tendency to form very insoluble aggregates, which are found in the brains of affected individuals. Such aggregates are soluble only in solvents containing both detergent and denaturant (typically 6 M guanidinium chloride); however it was shown that such aggregates can be solubilised by cationic dendrimers, such as PEI-, PPI- and PAMAM dendrimers, higher generation (> G3) dendrimers being the most efficient and influenced by the number of surface amino groups. PAMAM dendrimers having hydroxy groups at their surface (PAMAM-OH) and linear polymers had no or very minor effects. The effect was seen at surprisingly low concentrations (7 μg ml^{-1} or below) on aggregate producing neuroblastoma cells and took place with no cytotoxicity.[7] Other types of compounds capable of dissolving already formed aggregates of the prion protein have not been described.

7 Dendrimers in vaccines

It is well-established that small molecular weight substances (*e.g.* peptides) are not very immunogenic. *i.e.* no or a weak immune response (including antibody formation) is induced upon their injection into a recipient host. However, this problem can be overcome by increasing the molecular weight of the substance in question either by polymerisation or by coupling it to a multifunctional, high molecular weight carrier, traditionally a naturally-derived protein.[19] For the preparation of highly defined, reproducible immunogens, *e.g.* for human vaccine uses, other types of carriers are highly desirable and in this respect, dendrimers have emerged as useful since they can act as multivalent and well-defined carriers for antigenic substances by coupling of antigen molecules to the surface functional groups of the dendrimer.

Examples of dendrimer–peptide compounds being used for vaccine and immunization purposes include the multiple antigenic peptide (MAP) dendrimer system pioneered by Tam and coworkers,[14,143,144] which can be synthesised with defined mixtures of B- and T-cell epitopes,[145] either synthesized by stepwise peptide synthesis on the branches of the MAP or by segment coupling of peptide fragments by various methods.[143] By far, most of the reported immunizations with MAP constructs have been performed with traditional adjuvants as *e.g.* in the work by Moreno and coworkers[146] in which aluminium hydroxide, Freund's adjuvant, and a saponin adjuvant (QS-21) were tested for the ability to induce antibodies together with a MAP structure containing *Plasmodium falciparum* T- and B-cell stimulatory peptides. Using cancer related peptides, Ota and coworkers showed that MAPs were processed in antigen-presenting cells in the same way as antigens derived from intracellular pathogens (*e.g.* viruses), thereby providing a powerful immune response, including cytotoxic T-cells.[147]

The MAP construct is a wedgelike, asymmetrical dendrimer type formed by building successive generations of lysine residues acylating the α- and ε-amino groups of the preceding lysine residues. This results in a structure displaying an equal number of non-equivalent α- and ε-primary amino groups that can be coupled to a small molecular weight antigen of interest, with the purpose of rendering the antigen immunogenic and obviating the need to use carrier proteins. The most preferred MAP-structures for vaccination purposes are tetra- or octameric. By using orthogonal protection strategies, different types of antigenic moieties may be coupled in a controlled fashion to the same MAP carrier, Fig. 21.

A simple peptide carrier is based on a tetrameric MAP dendrimer in which cyclised antigenic peptides have been coupled and in which lipidic moieties are present in the core. Although no immunological data have yet been presented it is stated that this

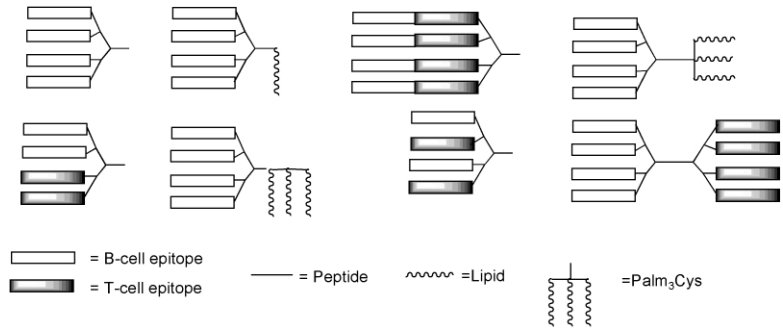

Fig. 21 Schematic depiction of different MAP-designs comprising different peptides representing T- and B-cell epitopes, respectively and showing different ways to organise these elements in MAP structures. Also shown is the possibility of including fatty acids into such structures either as single, straight chain alkyls or as the specific tripalmitatecysteinyl structure (*N*-α-palmitoyl [2,3-bis(palmitoyloxy)propyl]cysteine.

construct was a very promising foot and mouth disease vaccine candidate.[148]

MAP structures have been used in a large number of studies for producing peptide-specific antibodies[143] and are also being developed for vaccine use, a MAP-based malaria vaccine being now in phase I human trials.[146,149,150]

The MAP carrier has also been used for the preparation of non-peptide (carbohydrate, haptens) antigens for vaccination purposes. An example of the MAP system for glycoimmunogens is the Tn-antigenic dendrimer studied by Bay and coworkers[151] where a tetrameric core structure is derivatised with the Tn-antigen and with a Th-cell stimulatory peptide and was shown to react with monoclonal antibodies against Tn, Fig. 22. A different version of this, containing trimeric Tn-building blocks was later shown to be immunogenic,[152] and useful for active immunization against colon carcinomas in BALB/c mice, using alum as the adjuvant. As the mono-Tn analogue was less efficient than the tri-Tn analogue and as a linear analogue containing two tri-Tn moieties was also less efficient, it was concluded that the precise spatial arrangement and clustering of the Tn-epitope was very important for the immunogenicity. G5-PAMAM (Starburst™) dendrimers have been applied as carriers of the Tn-antigen and the resulting glycoconjugates were tested as vaccine candidates in comparison with a carrier protein (ovine serum albumin) conjugated to a monomer, dimer or trimer of the Tn-antigen. It was found that the Tn-antigen–dendrimer conjugates elicited no antibody response, and hence no immunogenicity, whereas Tn-antigen conjugated with a carrier protein or lipopeptide gave rise to antibody responses. The Tn-dimer lipopeptide conjugate also gave rise to IgG antibodies.[153]

Another peptide carrier system which is not dendrimeric *per se* but becomes a dendrimeric structure upon derivatisation with peptides is the nondendritic peptide carrier by Heegaard and coworkers,[154] in which the attachment points for the peptide branches are designed to space the attached peptides in an optimal fashion and to allow the peptide backbone to attain some structure in aqueous buffer, lending a certain degree of conformational definition to the whole complex and thereby supporting structural trends in the attached peptides. This phenomenon of organisationally induced structure has previously been demonstrated by Tuchscherer and coworkers[155] in the so-called template assisted synthetic peptides in which four identical peptides are coupled to a tetrafunctional, cyclic template, leading to an increased conformational definition of the peptides, compared to the peptides alone. This peptide construct was shown to be immunogenic in the absence of adjuvant and furthermore, in contrast to MAP, had high aqueous solubility.[154]

McGeary and coworkers have prepared carbohydrate-based (glycolipid) dendrimers as potential carriers for peptide antigens, utilising the multihydroxy functionalities of a single monosaccharide as the basis for multimeric presentation of antigens and showing the applicability of solid phase synthesis for this purpose.[156] Although no actual peptide–dendrimer constructs were

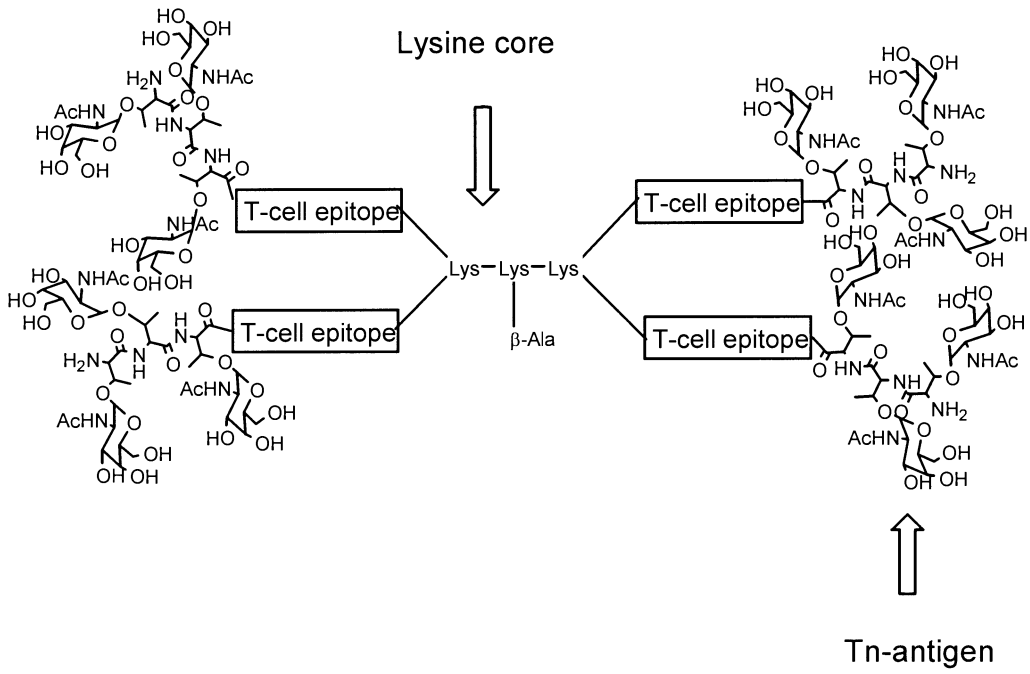

Fig. 22 Structure of a Tn-antigen based MAP immunogen comprising peptides representing T-cell stimulating epitopes in addition to trimeric Tn-epitopes (Galactose-threonine),[151] the T-cell epitope peptide being Lys-Leu-Phe-Ala-Val-Trp-Lys-Ile-Thr-Tyr-Lys-Asp-Thr (from the poliovirus VP1 protein).

synthesized and no immunization experiments were done, the use of carbohydrate functionalities for multimeric presentation of antigens is clearly warranted, and the possibility of including such structures into glycodendrimers (*vide supra*) for immunogen construction is presented.

Baek and coworkers explicitly claim that multimeric carbohydrate moieties as presented in glycodendrimers are non-immunogenic.[129] These and other examples indicate that multimericity is not enough by itself to render carbohydrates immunogenic, somewhat in contrast to peptide antigens (*vide supra*).

The use of dendrimers as adjuvants has been described by Rajananthanan and coworkers.[62] Here, the ability of different aggregate formulations to act as adjuvants is studied. Adjuvants are substances which augment the immune response to an antigen when both are administered together, and such compounds are virtually indispensable for the manufacture of efficient vaccines. Rajananthanan compares two glycolipid-containing aggregates with a G5-PAMAM dendrimer (5 nm diameter). The idea is, that amphilicity is related to protein carrying potential and thereby adjuvanticity. As such, the glycolipids of this study were to be expected to be much more amphiphilic than the dendrimer and moreover, they were meticulously formulated with various other components to prepare multimolecular complexes with non-covalently entrapped antigen *ad modum* Iscoms†.[157] However, when testing immunogenicity in mice with a standard protein antigen (ovalbumin), mixing antigen and dendrimer increased the immune response above that seen when administering the antigen alone, reaching titres in the 10^5 range being 10 times the titres reached with the antigen alone.

Wright claims that G3-PAMAM and other mid-generation dendrimers can be used as adjuvants for vaccine purposes when used in a dilution which ensures the absence of toxicity.[158]

Conclusion

Dendrimers are extremely well-defined, globular, synthetic polymers with a number of characteristics which make these polymers useful in biological systems. Dendrimers have unique properties enabling them to respond to changes in solvent conditions in a predictable manner and can easily be modified to act as highly specific binders of various biological substances. Dendrimers can be tailored or modified into biocompatible compounds with low cytotoxicity and high biopermeability *etc*. In addition, dendrimers are manufactured in high purities with few structural defects, and are easily analysed by standard methods as mass spectrometry, infrared spectroscopy and NMR spectroscopy.

As described, a number of dendrimer types are now commercially available and have already found use as drug candidates for receptor–ligand interactions, drug carriers for conferring biosurvival, membrane permeability and targeting, and have found wide use as carriers for vaccine antigens as well. Furthermore, dendrimers have proven very useful as scaffolds in the design of biosensors, imprinting scaffolds and artificial receptors (specific host–guest interactions).

It is to be expected that new dendrimer structures will continue to be developed, and more types of dendrimers will be manufactured to a high degrees of perfection and will become commercially available. Finally, new methods and strategies for synthesising, modifying and derivatising dendrimers will be developed, enabling specific tailoring of binding motifs, charge density *etc*.

This will add a new degree of sophistication to well-known drugs and reagents, as well as creating entirely new classes of drugs and bioactive substances based on these macromolecular, yet beautifully simple, structures.

† Iscoms are virus-like immunostimulating complexes consisting of lipid, adjuvant and antigen.

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