Design of functional dendritic polymers for application as drug and gene delivery systems

Review Article

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Key words: Dendrimers, Hyperbranched Polymers, Dendritic Polymers, Nanocarriers, Drug Delivery System, Gene Delivery

Abbreviations: Adriamycin, (ADR); arginine-grafted-PAMAM dendrimer, (PAMAM-Arg); Asialo-glycoprotein, (ASGP); betamethasone dipropionate, (BD); betamethasone valerate, (BV); Boron Neutron Capture Therapy, (BNCT); diaminobutane poly(propylene imine) dendrimer, (DAB); diaminobutane poly(propylene imine) fourth generation dendrimer functionalized with 6 guanidinium groups, (DAB-G6); diaminobutane poly(propylene imine) fourth generation dendrimer functionalized with 12 guanidinium groups, (DAB-G12); Dynamic Light Scattering, (DLS); epidermal growth factor, (EGF); green fluorescent protein, (GFP); injected dose, (ID); hyperbranched poly(ethylene imine), (PEI); hyperbranched polyglycerol, (PG); Isothermal Titration Calorimetry, (ITC); L-lysine grafted-PAMAM dendrimer, (PAMAM-Lys); methotrexate, (MTX); methoxypoly(ethylene glycol)-isocyanate, (PEG-isocyanate); PEGylated diaminobutane poly(propylene imine) imide dendrimer with 4 PEG chains, (DAB-4PEG); PEGylated diaminobutane poly(propylene imine) imide dendrimer with 8 PEG chains, (DAB-8PEG); PEGylated polyglycerol, (PG-PEG); PEGylated-Folate polyglycerol, (PG-Folate); Phosphate Buffer Saline, (PBS); poly(amidoamine) dendrimer, (PAMAM); poly(amidoamine) dendrimer with terminal hydroxyl groups, (PAMAM-OH); poly(ethylene imine)-poly(ethylene glycol)-folate, (PEI-PEG-FOL); poly(ethylene glycol), (PEG); poly(ethylene glycol) monomethyl ether, (M-PEG); poly(propylene imine) dendrimer, (PPi); primaquine phosphate, (PP); pyrene, (PY); quaternized poly(amidoamine) dendrimer with terminal hydroxy groups, (QPAMAM-OH); tamoxifen, (TAM)

Received: 28 November 2005; Accepted: 10 February 2006; electronically published: March 2006

Summary

The present review deals with the design and preparation of functional and multifunctional dendrimeric and hyperbranched polymers (dendritic polymers), in order to be employed as drug and gene delivery systems. In particular, using as starting materials known and well-characterized basic dendritic polymers, the review discusses the kind of structural modifications that these polymers were subjected for preparing nanocarriers of low toxicity, high encapsulating capacity, specificity to certain biological cells and transport ability through their membranes. Due to the great number of external groups of dendritic polymers either functionalization or multifunctionalization can occur, providing products that fulfill one or more of the requirements that an effective drug carrier should exhibit. A common feature of these dendritic polymers is the exhibition of the so-called polyvalent interactions, while for the multifunctional derivatives a number of targeting ligands determines specificity, other groups secure stability in biological milieu, while others facilitate their transport through cell membranes. In addition, for gene delivery applications these multifunctional systems should be or become cationic in the biological environment for the formation of complexes with the negatively charged genetic material.

I. Introduction

Dendrimers are prepared by tedious synthetic procedures (Bosman et al, 1999; Schlüter and Rabe, 2000; Fréchet and Tomalia, 2001; Newkome et al, 2001) and they are nanometer-sized, highly branched and monodisperse macromolecules with symmetrical architecture. They consist of a central core, branching units and terminal functional groups. The core and the internal units determine the environment of the nanocavities and consequently their solubilizing or encapsulating properties, whereas, the external groups their solubility and chemical behaviour. On the other hand, hyperbranched polymers (Inoue, 2000), including the extensively investigated hyperbranched polyether polyols or polyglycerols (Sunder et al, 1999a, b; 2000a,b; Haag, 2001; Frey and Haag, 2002; Siegers et al, 2004) are conveniently prepared. Hyperbranched polymers are non-
symmetrical, highly branched and polydisperse macromolecules, while their main structural feature, also common to dendrimers, is that they exhibit nanocavities. These two types of polymers are called dendritic polymers, the nanocavities of which, depending on their polarity, can encapsulate various molecules, including active drug ingredients. The external groups of dendritic polymers can be modified providing a diversity of functional materials (Vögtle et al, 2000) that can be employed for various applications.

Within this context, commercially available or custom-made dendrimeric or hyperbranched polymers can be functionalized for being used as effective systems for drug (Liu and Fréchet, 1999; De Jes-s et al, 2002; Stiriba et al, 2002; Beezer et al, 2003; Gillies and Fréchet, 2005) and gene (Bielinska et al, 1999; Luo et al, 2002; Ohsaki et al, 2002) delivery. Since more than one type of groups can be introduced at the surface of the dendritic polymers, these systems are characterized as multifunctional as shown in Figure 1. Each type of groups plays a specific role in the application of multifunctional dendritic polymers as drug delivery systems. Thus, specificity for certain cells can be accomplished by attaching targeting ligands at the surface of dendritic polymers, while enhanced solubility, decreased toxicity, biocompatibility, stability and protection in the biological milieu can be achieved by the functionalization of the end groups of dendritic polymers, for instance, with poly(ethylene glycol) chains (PEG). The function of PEG-chains is crucial for modifying the behaviour of drug themselves or of their carriers (Noppl-Simson and Needham, 1996; Ishiwata et al, 1997; Liu et al, 1999; Liu et al, 2000; Veronese, 2001; Roberts et al, 2002; Pantos et al, 2004; Vandermeulen and Klok, 2004).

Targeting ligands are complementary to cell receptors (Cooper, 1997; Lodish et al, 2000) and induce the attachment of the nanocarrier to the cell surface. This binding is further enhanced due to the so-called polyvalent interactions (Mammen et al, 1998; Kitov and Bundle, 2003) attributed to the close proximity of the recognizable ligands on the limited surface area of the dendritic molecules. On the other hand, as it has long been established with liposomes (Lasic and Needham, 1995; Crosasso et al, 2000; Needham and Kim, 2000; Silvander et al, 2000), PEG-chains may prolong the circulation of liposomes in biological milieu. Transport through the cell membrane can also be facilitated by the introduction of appropriate moieties at the surface of the dendritic polymers. In addition, modification of the internal groups of dendrimers affects their solubilizing character, making, therefore, possible the encapsulation of a diversity of drugs. In this connection, cationization of dendrimers, and particularly of their external groups, facilitates their application as gene transfer agents (Bielinska et al, 1999; Luo et al, 2002; Ohsaki et al, 2002) due to formation of DNA-Dendritic Polymer complexes.

Monofunctional dendritic drug carriers do not simultaneously show the desired properties that multifunctional derivatives exhibit. Thus, in this review, starting from selected monofunctional systems and specifically from the dendrimeric compounds poly(amidoamine), PAMAM, and diaminobutane poly(propylene imine), DAB, and also from the hyperbranched polymers polyglycerol, PG and poly(ethylene imine), PEI, (Figure 2) a stepwise design of multifunctional systems will be discussed, aiming at obtaining appropriate nanocarriers for drug delivery and gene transfection. This review is by no means exhaustive and only selected examples will be discussed highlighting on work performed recently in our laboratory. The objective of this review is to illustrate the effectiveness of the strategy of molecular engineering, applied on dendritic surfaces, to prepare drug carriers with desired properties.

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**Figure 1.** Schematic representation of a multifunctional dendrimer.

- = Branching point  
○ = Terminal group  
□ = Recognizable group  
☒ = Protective coating
II. Drug carriers: from monofunctional to multifunctional dendrimers

In an example on molecular engineering of PAMAM surface, poly(ethylene glycol) monomethyl ether (M-PEG), having an average molecular weight of 550 or 2000, was attached at the terminal amino groups of the third and fourth generation polymers as shown in Figure 3. Inside the nanocavities of the so-prepared PEGylated dendrimers, Adriamycin, ADR, or Methotrexate, MTX, anticancer drugs (Figure 4) were encapsulated (Kojima et al, 2000). As the amount of ADR employed for encapsulation inside these PEGylated dendrimers increased, the number of ADR molecules associated with the dendrimer increased and finally reached a plateau. Depending on the generation, the maximum number of ADR molecules encapsulated per dendrimer i.e. by the M-PEG(550)-G3, M-PEG(2000)-G3, M-PEG(550)-G4, and M-PEG(2000)-G4 dendrimeric derivatives are ca. 1.2, 2.3, 1.6, and 6.5, respectively, as shown in Figure 5. Thus, the encapsulation ability varied for these PEG-dendrimers and it was found to depend on the molecular weight of PEG-chains and also on dendrimers’ generation.

PAMAM has a basic interior and, therefore, it is possible to encapsulate MTX, which is acidic, since it bears two carboxyl groups. The number of MTX molecules associated with one dendrimer molecule, as a function of the MTX/dendrimer ratio during loading is shown in Figure 6. As it was observed in the

Figure 2. Chemical structure of dendrimeric compounds.
Figure 3. Preparation and structure of M-PEG PAMAM dendrimer of the third generation. Reproduced from Kojima et al, 2000 with kind permission from the authors and American Chemical Society.

Figure 4. Chemical structure of the anticancer drugs adriamycin, ADR, and Methotrexate, MTX

Figure 5. Encapsulation of ADR by M-PEG(550)-attached (open symbols) or M-PEG(2000)-attached (closed symbols) PAMAM G3 (●●) and G4 (●●) dendrimers. The number of ADR encapsulated per dendrimer is shown as a function of the ADR/dendrimer molar ratio during loading. Reproduced from Kojima et al, 2000 with kind permission from American Chemical Society.
encapsulation of ADR, the number of MTX molecules associated with the modified dendrimer increased with increasing amount of MTX employed during loading, and finally reached a constant value. The maximum numbers of MTX molecules associated with the M-PEG(550)-G3, M-PEG(2000)-G3, M-PEG(550)-G4, and M-PEG(2000)-G4 dendrimers are approximately 10, 13, 20, and 26 mol/mol of dendrimer, respectively. Apparently, the number of the encapsulated drugs by the PEGylated dendrimers increased when MTX was used instead of ADR. Since these drugs have similar molecular weights, this result suggests that the electrostatic interaction from the acid-base interaction between the dendrimer and MTX molecules results in an enhanced encapsulation of MTX by these dendrimers. As it was the case with ADR encapsulation, the number of MTX encapsulated by the dendrimer was affected both by generation of the PAMAM and by the chain length of the M-PEG.

Release experiments performed in PBS buffer (Phosphate Buffer Saline) showed that ADR was readily released from the modified dendrimers. Apparently, hydrophobic interaction between ADR and the dendrimer is not strong enough to retain the drug in the interior of the PAMAM dendrimeric moiety. The release of MTX from the M-PEG-functionalized dendrimers was also investigated by the same method. The time dependency of MTX concentration in the outer phase during the dialysis is shown in Figure 7. Apparently, the MTX concentration in the outer phase increased at a slower rate when MTX was encapsulated in the M-PEG-attached dendrimer than in the case of free MTX. This indicates that MTX was gradually released from the modified dendrimer. As mentioned above, MTX was electrostatically bound to the dendrimeric interior and, therefore, dissociation of MTX from the dendrimer was suppressed to some extent. However, when the dialysis was performed in the presence of 150 mM NaCl, no difference in the release rate was observed between MTX encapsulated in the M-PEG-attached dendrimer and free MTX. In this case, MTX can dissociate readily from the dendrimer because the electrostatic interaction is weakened by the shielding effect of Na$^+$ and Cl$^-$ (Kojima et al, 2000).

Effective solubilization of hydrophobic drugs was, however, achieved with another PEGylated dendritic system (Sideratou et al, 2001), which is analogous to the one previously discussed. PEGylation of dendrimers was performed under facile experimental conditions by the interaction of methoxypoly(ethylene glycol)-isocyanate (PEG-isocyanate) with the external primary amino groups of DAB dendrimers of fifth generation, as shown in Figure 8. Two different PEGylated dendrimeric derivatives were prepared i.e. the DAB-4PEG (weakly PEGylated) and DAB-8PEG (densely PEGylated). In this manner, the role of PEG-coating on encapsulation and release properties was possible to be assessed.

Comparison of solubilizing ability of the parent and PEGylated DAB dendrimers is shown in Table 1. For this purpose, betamethasone valerate, BV, and betamethasone dipropionate, BD, were used as active drug ingredients (Figure 9). These anti-inflammatory corticosteroids are practically water insoluble and it is, therefore, necessary to encapsulate these compounds in a water-soluble carrier for facilitating their use as drugs. The concentration of encapsulated betamethasone derivatives was significantly increased in PEGylated dendrimers. Thus, for DAB-8PEG the loading was 13 and 7 wt.%, for BV and BD, while for DAB-4PEG was 6 and 4 wt.%, respectively. The observed solubility increase was attributed to an additional solubilization of the compounds in PEG-chains by which the dendrimers are coated. This is also verified by the fact that upon protonation they remain solubilized in PEG-chains environment. As expected, by increasing dendrimer concentration, solubilization of drugs analogously increases to a certain limit.
Figure 7. Release of MTX from the M-PEG(2000)-attached G4 dendrimer. The MTX-loaded M-PEG(2000)-G4 dendrimer (○, ▲) or free MTX (△, ▲) dissolved in 1 mM Tris-HCl-buffered solution (pH 7.4) containing (open symbols) or not containing (closed symbols) 150 mM NaCl and dialyzed against the same solution. The time course of MTX concentration in the outer phase during the dialysis is shown in the figure. Reproduced from Kojima et al, 2000 with kind permission from American Chemical Society.

Figure 8. Preparation and structure of PEGylated DAB dendrimer of the fifth generation functionalized with 4 or 8 PEG chains.

For a detailed investigation of the solubilization site and release properties of these PEGylated dendrimers the hydrophobic pyrene was employed. This is a very sensitive probe and it is used as a model compound when drugs cannot offer this type of information. By employing the well-known $I_1/I_3$ fluorescence intensity ratio, which probes the polarity of the medium (Thomas, 1980), it was found that pyrene is solubilized both in the core and in PEG-chains. In addition, upon protonation of the loaded PEGylated dendrimer, pyrene is not released in the bulk aqueous phase as judged again by the $I_1/I_3$ ratio and fluorescence intensity ($F/F^0$) results. This is attributed to the fact that as pyrene is leaving the core it is possible to be solubilized inside PEG-chains, as shown schematically in Figure 10. The results of $I_1/I_3$ fluorescence intensity ratio indicate that pyrene is neither solubilized in the bulk water phase nor in the interior of the dendrimer. Normally, one would expect release of pyrene in water, since, due to protonation, the environment of the nanocavities becomes polar and, therefore, the hydrophobic pyrene cannot remain solubilized. In addition, protonated tertiary amino groups of the core do not exhibit anymore the property to form charge-transfer complexes with pyrene (Sideratou et al, 2000) and, therefore, encapsulation of the pyrene is no longer favoured. It should, however, be noted that complete release of pyrene can be achieved upon exhaustive dilution of the PEGylated dendrimer. The same behaviour was observed for the hydrophobic drugs BV and BD. In conclusion, the enhanced solubilization of these drugs in PEGylated dendrimers secures their application as promising controlled release drug delivery systems.

In another recent report, extending the previous work, a novel multifunctional dendrimeric carrier was designed (Paleos et al, 2004) based on diaminobutane poly(propylene imine) dendrimer of the fifth generation. The synthetic procedure of this derivative is shown in Figure 11. This carrier is intended to simultaneously address issues such as stability in the biological milieu, targeting and very possibly transport through cell membranes. For this purpose, in addition to surface protective poly(ethylene glycol) chains, guanidinium moieties were introduced as targeting ligands. In addition, the accumulation of guanidinium groups at the surface of the dendrimer may also facilitate its transport ability. The functional groups were covalently attached at the
dendrimeric surface and it was possible to secure, in principle, desired drug delivery properties due to: a. Protection of the carrier because of the coverage of the dendrimeric surface with poly(ethylene glycol) chains, b. Recognition ability towards complementary moieties; surface guanidinium groups secure the facile interaction with acidic receptors including the biologically significant carboxylate and phosphate groups. Combined electrostatic forces and hydrogen bonding are exercised making this interaction thermodynamically favorable (Hirst et al, 1992), c. Possibility of encapsulation and release of active drug ingredients from the nanocavities, which can be tuned by environmental changes (Sideratou et al, 2001), d. Complexation with DNA for gene therapy applications, e. The occurrence of polyvalency interactions, associated with enhanced binding, due to the accumulation of recognizable moieties on the limited surface area of the dendrimer as schematically illustrated in Figure 12, f. The expected decrease of toxicity due to the facile modification of the toxic amino groups (Malik et al, 2000).

Table 1. Comparative solubility of pyrene (PY), betamethasone valerate (BV) and betamethasone dipropionate (BD) in parent DAB and PEGylated derivatives. Reproduced from Sideratou et al, 2001 with kind permission from Elsevier.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[dendrimer]/M</th>
<th>[PY]/M</th>
<th>[BV]/M</th>
<th>[BD]/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB</td>
<td>5 x 10^{-5}</td>
<td>2.15 x 10^{-6}</td>
<td>2.95 x 10^{-5}</td>
<td>1.84 x 10^{-5}</td>
</tr>
<tr>
<td>DAB-8PEG</td>
<td>5 x 10^{-5}</td>
<td>5.40 x 10^{-5}</td>
<td>3.85 x 10^{-4}</td>
<td>2.56 x 10^{-4}</td>
</tr>
<tr>
<td>DAB-4PEG</td>
<td>5 x 10^{-5}</td>
<td>2.14 x 10^{-3}</td>
<td>2.05 x 10^{-3}</td>
<td>1.25 x 10^{-4}</td>
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<tr>
<td>DAB-8PEG</td>
<td>5 x 10^{-4}</td>
<td>8.75 x 10^{-3}</td>
<td>3.65 x 10^{-2}</td>
<td>1.87 x 10^{-3}</td>
</tr>
<tr>
<td>DAB-4PEG</td>
<td>5 x 10^{-4}</td>
<td>5.25 x 10^{-3}</td>
<td>1.70 x 10^{-3}</td>
<td>1.09 x 10^{-3}</td>
</tr>
</tbody>
</table>

Figure 9. Chemical structure of betamethasone valerate, BV, and betamethasone dipropionate, BD

Figure 10. Schematic representation of the solubilization of pyrene in PEGylated dendrimers. Reproduced from Sideratou et al, 2001 with kind permission from Elsevier BV.
For evaluating the loading capacity and release properties of the above multifunctional dendrimer, pyrene (PY) and betamethasone valerate (BV), were used as model compounds. The dendrimeric derivative encapsulated significantly higher concentrations of the above compounds compared to the parent dendrimer, as determined by UV spectroscopy and shown in Table 2. This is particularly significant for betamethasone valerate, of which seven molecules are solubilized per dendrimeric molecule. As previously mentioned (Sideratou et al, 2001), this was attributed to the presence PEG-chains. Additionally, in the case of betamethasone valerate the loading capacity is 11 wt% for the multifunctional dendrimer, i.e. almost double compared to the loading capacity of the simply PEGylated dendrimer (6 wt%) and more than five times compared to the loading capacity of the parent dendrimeric solution (1.7 wt%) (Sideratou et al, 2001). This is quite beneficial for its use as drug delivery system and it can only be attributed to the other two functional groups introduced at the surface of the multifunctional derivative. As it will be discussed below, they may act synergistically enhancing solubilization of betamethasone valerate.

Pyrene, as in the previous work (Sideratou et al, 2001), was in first place employed as model hydrophobic compound for probing the solubilization properties of the multifunctional dendrimer. For this reason fluorescence intensity ($F/F_0$) changes and $I_1/I_3$ ratio were monitored, which are sensitive parameters and their values depend on the medium of solubilization of the probe. These parameters were monitored by a titration-like addition of the dendrimer to an aqueous pyrene solution. A significant quenching of fluorescence intensity ($F/F_0$) was observed and the $I_1/I_3$ ratio decreased (Figure 13). Fluorescence quenching was attributed (Sideratou et al, 2001) to the formation of a charge-transfer complex between pyrene and tertiary amino groups, as evidenced by the appearance of a weak exciplex fluorescence centered at approximately 485 nm (Lakowicz, 1983). As the concentration of the dendrimer increases, the $I_1/I_3$ ratio decreases to a value of about 0.90, which is close to the one observed in the hydrophobic environment usually encountered in the

**Figure 11.** Reaction scheme for the synthesis of a multifunctional dendrimeric derivative. Reproduced from Paleos et al, 2004 with kind permission from American Chemical Society.

**Figure 12.** Schematic representation of a dendrimer exhibiting polyvalent properties.
conventional micelles. Thus, pyrene is mainly incorporated inside the nanocavities of the dendrimer, in order to avoid contact with the hydrophilic external groups.

The release of the active ingredient from the dendrimer when it reaches the target site enhances its bioavailability and efficacy. In addition, drug release from endosomal compartment appears a limiting factor for several targeted drug delivery formulations (Boomer et al., 2003). These requirements impose the need for developing drug delivery systems in which the release of drug can be triggered by appropriate stimulus. For this purpose pH-triggered, enzymatic, thermal and photochemically induced processes have been reported (Boomer et al., 2003). For instance low pH within endosomal and ischemic tissue environments renders acid triggerable delivery systems attractive for controlled release.

The multifunctional poly(propylene imine) dendrimers prepared, due to the presence of tertiary amino groups in their core fulfill at least one of these requirements, i.e. being pH responsive (Sideratou et al., 2000; Sideratou et al., 2001; Paleos et al., 2004). As found in the previous experiment pyrene is solubilized in the interior of dendrimer and also within PEG chains, while upon protonation of tertiary amines of the nanocavities pyrene is repositioned in the PEG coat.

For achieving the release of the encapsulated pyrene from the PEG protective coat another method has, therefore, to be explored. We were prompted to use aqueous sodium chloride solution for triggering pyrene release since, as it has been established in independent studies (Wang et al., 2000; Bogan and Agnes, 2002), ions of alkali metals cationize poly(ethylene glycol) moieties through complexation. The designed multifunctional dendrimer, due to the attachment of PEG chains at its surface, is susceptible to analogous interactions and, therefore, it could be possible for metal cations to replace solubilized pyrene releasing it to the bulk aqueous phase. Indeed, by titrating dendrimeric solutions with sodium chloride solution, pyrene was released and dispersed in the bulk solution in the form of crystallites. The isolated crystallites were indentified by $^1$H NMR and proved to be pure pyrene.

The two-step triggered release from the multifunctional dendrimer was also investigated using the lipophilic drug betamethasone valerate. Release of the drug with hydrochloric acid has not been observed since betamethasone valerate remained solubilized within the dendrimeric environment and preferably within the poly(ethylene glycol) chains. Betamethasone valerate encapsulated in the multifunctional dendrimer was completely released upon addition of sodium chloride as shown in Figure 14. However, within the concentration

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**Table 2.** Comparative solubility of pyrene (PY) and betamethasone valerate (BV) in the parent fifth generation DAB and multifunctional dendrimer. Reproduced from Paleos et al, 2004 with kind permission from American Chemical Society.

<table>
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<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$2.1_{\pm0.2} \times 10^{-5}$</td>
<td>0.021$_{\pm0.002}$</td>
<td>$2.5_{\pm0.4} \times 10^{-4}$</td>
<td>0.25$_{\pm0.04}$</td>
</tr>
<tr>
<td>Multifunctional</td>
<td>$2.5 \times 10^{-4}$</td>
<td>$1.9_{\pm0.08} \times 10^{-5}$</td>
<td>0.076$_{\pm0.002}$</td>
<td>$1.80_{\pm0.4} \times 10^{-3}$</td>
<td>7.20$_{\pm0.03}$</td>
</tr>
<tr>
<td>Dendrimer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Figure 13. Plot of $F/F^0$ and $I_1/I_3$ ratio as a function of the concentration of the multifunctional dendrimer. $F^0$ is the total fluorescence intensity of $6.81 \times 10^{-7}$ M aqueous solution of pyrene and $F$ is the measured fluorescence intensity at various dendrimer concentrations. Reproduced from Paleos et al, 2004 with kind permission from American Chemical Society.
range of the sodium cation present in extracellular fluids, i.e. 0.142 M, (Guyton and Hall, 2000) the betamethasone valerate was released in relatively small quantities. The gradually released betamethasone valerate from the multifunctional dendrimer formed crystallites in the aqueous medium as determined by light scattering. The precipitated material was analyzed with $^1$H NMR and its spectrum corresponded to that of betamethasone valerate. This finding should be considered when PEGylated dendrimers are used as drug delivery systems in experiments in vitro and in vivo, since sodium chloride in extracellular fluids and potassium chloride in intracellular environment can be complexed with PEG chains (Wang et al, 2000; Bogan and Agnes, 2002) affecting the overall release profile of the drug. Thus, the possibility of triggering drug release in the extracellular fluid, i.e. before endocytosis to the target-cells, should be taken into account when designing a targeted PEGylated drug delivery system.

The drug delivery effectiveness of analogous multifunctional dendrimers was modeled by investigating their interaction with multilamellar liposomes consisting of phosphatidylcholine/cholesterol/dihexadecyl phosphate (19:9.5:1) and dispersed in aqueous or phosphate buffer solutions (Pantos et al, 2005). The multilamellar liposomes bear the phosphate moiety as recognizable group. They were used as simple models before one resorts to the use of cells; after all liposomes are considered as the closest analogues to cells. On the other hand, poly(propylene imine) fourth generation dendrimers were functionalized with 6 (DAB-G6 ) or 12 (DAB-G12) guanidinium groups as targeting ligands, while the remaining toxic, external primary amino groups of the dendrimers were allowed to interact with propylene oxide affording the corresponding hydroxylated derivatives. The scheme of the reactions modifying the dendrimeric surface is shown in Figure 15. DAB-G0 dendrimer, which does not contain any guanidinium group was used as a reference compound. The so-prepared dendrimers were loaded with corticosteroid drugs, i.e. betamethasone dipropionate and betamethasone valerate for investigating their transfer to liposomes.

Microscopic, $\zeta$-potential, and Dynamic Light Scattering (DLS) techniques have shown that liposomes-dendrimers molecular recognition occurs leading to the formation of large aggregates at dendrimer/dihexadecyl phosphate molar ratios higher than 1:30, as visually observed with phase contrast optical microscopy. Calcein liposomal entrapment experiments demonstrate a limited leakage, i.e. less than 13%, following liposomes interaction with the modified dendrimers at 1:25 dendrimer/ dihexadecyl phosphate molar ratio. This indicates that the membrane of the liposomes remains almost intact during their molecular recognition with these dendrimers. Isothermal Titration Calorimetry (ITC) indicates that the enthalpy of the interaction is dependent on the number of the guanidinium groups present at the dendrimeric surface. Furthermore, the process is reversible and redispersion of the aggregates occurs by adding concentrated phosphate buffer.

The interaction between these drug-loaded dendrimers and multilamellar liposomes results in the transport of drugs from the dendrimeric derivatives to the ‘empty’ liposomes as summarized in Table 3. The experiments demonstrate that about 25% of BD or BV is present in the precipitated aggregates when DAB-G0 was used. When the guanidinylated dendrimers DAB-G6 and DAB-G12 were used, the amount of drugs in the precipitate increases substantially becoming about 60% and 80%, respectively.

![Figure 14](https://example.com/figure14.png)

Figure 14. Plot of the concentration of betamethasone valerate in a 2.50 x 10$^{-5}$ M dendrimeric solution as a function of added NaCl. Reproduced from Paleos et al, 2004 with kind permission from American Chemical Society.
These significant differences observed in the transport of drugs between guanidinylated and non-guanidinylated dendrimers can be attributed to the functionalization of the dendrimeric molecules. The presence of guanidinium groups at the external surface of the dendrimers results in an effective adhesion to the multilamellar liposomes as the ITC and DLS experiments demonstrated. As expected, when the interaction is taking place in 10mM phosphate buffer the drug present in the aggregates decreases slightly. In this case, the decrease of drug transport can be rationalized by the competitive interaction of the phosphate groups in bulk with the guanidinium dendrimeric groups leading to less effective adhesion with the multilamellar liposomes.

Upon the addition of concentrated phosphate buffer followed by the redispersion of the aggregates in the medium and the separation of the no-longer interacting dendrimers, drugs are still present in the obtained multilamellar liposomes. Determination of BD or BV in the multilamellar liposomes indicates that, in all cases, ca. 50% (Table 3) of the amount of drugs found in the aggregates before redispersion is still present, suggesting that they are located in the lipid bilayer, since their solubility in water is extremely low. Drug transport is induced by the use of guanidinylated dendrimers since drug transport values of about 40-45% were obtained in the case of DAB-G12, while only 12-15% was observed in the case of the non-guanidinylated derivative.

Carbohydrates, in general, being targeting ligands for selectins can be introduced at the external surface of dendrimers leading to the formation of targeted drug delivery systems. In a recent study (Bhadra et al, 2005), galactose surface-coated poly(propylene imine) (PPI) dendrimeric derivatives were prepared and loaded with primaquine phosphate (PP) (Figure 16), which is an antimalarial drug. Galactose functionalization was carried

![Figure 15. Functionalization of poly(propylene imine) dendrimer of the fourth generation including guanidinylation at the final step. Reproduced from Pantos et al, 2005 with kind permission from American Chemical Society.](image)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dendrimer</th>
<th>Drug transfer (%) in aggregates</th>
<th>Drug transfer (%)after redispersion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>Phosphate Buffer</td>
</tr>
<tr>
<td>BD</td>
<td>DAB-G0</td>
<td>24.4 ±2.4</td>
<td>19.8 ±1.2</td>
</tr>
<tr>
<td></td>
<td>DAB-G6</td>
<td>62.5 ±1.9</td>
<td>48.5 ±1.6</td>
</tr>
<tr>
<td></td>
<td>DAB-G12</td>
<td>84.5 ±2.1</td>
<td>68.4 ±1.5</td>
</tr>
<tr>
<td></td>
<td>DAB-G0</td>
<td>32.9 ±2.0</td>
<td>27.1 ±1.0</td>
</tr>
<tr>
<td>BV</td>
<td>DAB-G6</td>
<td>59.0 ±1.5</td>
<td>39.5 ±2.1</td>
</tr>
<tr>
<td></td>
<td>DAB-G12</td>
<td>78.1 ±2.3</td>
<td>57.5 ±2.0</td>
</tr>
</tbody>
</table>

Table 3. Drug transfer (%) from dendrimers to multilamellar liposomes in a) aggregates obtained after their interaction in water or in 10 mM phosphate buffer (pH 7.4) and b) multilamellar liposomes obtained following redispersion of the aggregates. Reproduced from Paleos et al, 2005 with kind permission from American Chemical Society.
out by a ring opening reaction followed by Schiff’s base reaction and reduction to secondary amine in sodium acetate buffer as shown in Figure 17.

Galactose had been shown to be a promising ligand for hepatocyte (liver parenchymal cells) targeting because liver cells possess a large number of the Asialo-glycoprotein (ASGP) receptors that can recognize the galactose units on the oligosaccharide chains of glycoproteins, or on chemically galactosylated drug carriers (Ashwell and Harford, 1982). The receptor-ligand interaction was known to exhibit a significant ‘cluster effect’ in which a polyvalent interaction results in extremely strong binding of ligands to the receptors.

The results obtained indicated that galactose coating of PPI systems increases the drug entrapment efficiency by 5-15 times depending upon dendrimers’ generation. Also galactose coating prolonged release up to 5–6 days as compared to 1-2 days for uncoated PPI. The hemolytic toxicity, blood level and hematological studies proved that these carriers are safer and suitable for sustained drug delivery. Blood level studies proved the suitability of the carriers in prolonging the circulations and delivery of PP to liver.

Figure 16. Chemical structure of primaquine phosphate.

Figure 17. Galactosylation of poly(propylene imine) dendrimer of the third to fifth generation.
Proceeding with further functionalization and employing a third generation PAMAM dendrimer as a starting compound, multifunctional dendrimers were prepared (Shukla et al, 2003). These derivatives, in addition to the protective PEG-chains they also bear the folate moiety at the end of poly(ethylene glycol) chain which can induce endocytosis into folate receptor-bearing cells (Sudimack and Lee, 2000; Hofland et al, 2002; Antony, 2004; Saharanjak and Mayor, 2004). The folate receptor is known to be significantly overexpressed over a wide variety of human cancers and, therefore, folate-mediated targeting has been widely applied with liposomes (Lee and Low, 1995; Lee and Huang, 1996; Gabizon et al, 2004), dendrimers (Kono et al, 1999; Konda et al, 2001; Shukla et al, 2003), various polymers and particles (Dauty et al, 2002; Dubé et al, 2002; Aronov et al, 2003; Zuber et al, 2003; Yoo and Park, 2004; Kim et al, 2005b; Liccari di et al, 2005; Wang and Hsiue, 2005) when used as drug delivery systems. In addition, the previously functionalized dendrimer 12 to 15 decaborate clusters were covalently attached, which can be used for the treatment of cancer in Boron Neutron Capture Therapy (BNCT) requiring the selective delivery of $^{10}$B to cancerous cells within a tumor. Varying number of PEG chains of varying length were linked to these boronated dendrimers to reduce hepatic uptake. Among all prepared combinations, boronated dendrimers with 1-1.5 PEG$_{2000}$ units exhibited the lowest hepatic uptake in C57BL/6 mice (7.2-7.7% injected dose (ID)/g liver).

Two folate receptor-targeted boronated third generation poly(amidoamine) dendrimers were prepared, the one shown in Figure 18, one containing ~15 decaborate clusters and ~1 PEG$_{2000}$ unit with a folic acid moiety attached to the distal end, while the other was containing ~13 decaborate clusters, ~1 PEG$_{2000}$ unit and ~1 PEG$_{800}$ unit with folic acid attached to the distal end. In vitro studies using folate receptor (+) KB cells demonstrated receptor-dependent uptake of the latter folic acid-functionalized derivative. Biodistribution studies with this derivative in C57BL/6 mice bearing folate receptor (+) murine 24JK-FBP sarcomas resulted in selective tumor uptake (6.0% ID/g tumor), but also high hepatic (38.8% ID/g) and renal (62.8% ID/g) uptake (Table 4), indicating that attachment of a second PEG unit and/or folic acid may adversely affect the pharmacodynamics of this conjugate.

In conclusion, the optimal modification of Boronated dendrimers as well as of dendrimers in general with PEG chains for reducing reticuloendothelial system affinity appears to be a highly complex process that depends on a variety of factors requiring extensive evaluation. The folic acid functionalized PEGylated G3-Boronated Dendrimer showed significantly increased tumor selectivity compared with non-PEGylated Boronated Dendrimeric-antibody and Boronated Dendrimer-EGF (Epidermal growth factor) conjugates previously evaluated for potential application in BNCT (Barth et al, 1994; Yang et al, 1997). However, the hepatic and renal uptake of this conjugate was very high.

III. Drug carriers: from monofunctional to multifunctional hyperbranched polymers

Based on polyglycerol (PG) and also on poly(ethylene imine) (PEI), pH-responsive molecular carriers were prepared (Krämer et al, 2002) through appropriate functionalization. The concept of pH-responsive carriers may have potential application for selective drug delivery in tissues of a lower pH value (for example, infected or tumor tissue). Polyglycerol and poly(ethylene imine), which are commercially available, are randomly branched but have defined dendritic structures with a degree of branching 60 to 75%. Functionalization of these dendritic polymers was achieved through a facile condensation reaction between the 1,2-diol or NH$_2$ moieties at their external surface and various carbonyl compounds as shown in Figure 19. Several dendritic structures originating from these reactions have been prepared, differing in the following: a. the type and molecular weight of the core polymer, b. the structure of the attached peripheral shell and c. the degree of alklylation.

The loading capacities (number of encapsulated congo red per polymeric nanocarrier) of dendritic polymers together with their structural features are shown in Table 5. It was found that a minimum core size (ca. 3000 gmol$^{-1}$) and a highly branched architecture are required for successful encapsulation of the guest molecules. For efficient encapsulation the degree of alklylation should be about 45-50% and the alkyl chains should have a minimum length (>C10). For example, the conversion of the terminal groups in polyglycerol, PG (21 000 gmol$^{-1}$) with a C16 aldehyde (PGe) containing one alkyl chain per diol unit results in an effective degree of alkyl functionalization of 25% (Table 5) and a poor encapsulation capacity (0.15 congo red molecules). With the same PG core (21 000 gmol$^{-1}$), the ketal functionalized carrier, PGb, with two alkyl chains per diol unit and 45% effective alkyl functionalization (Table 5) can encapsulate up to 13 congo red molecules. A higher degree of ketal functionalization (PGc: 55%, Table 5) indicates an optimal shell density of 45-50%. The exact determination of the encapsulation capacities for the amine based poly(ethylene imine) carriers was complicated because of the hydrolytic sensitivity of the imine-bound peripheral shell in the PEI-based systems, for instance in PEIb (Table 5). To avoid hydrolysis the dye was directly encapsulated from the solid/organic solution interface.

The complexation of an antitumor drug, mercaptourine, several oligonucleotides, as well as bacteriostatic silver compounds (for example, AgI salts and Ag$^0$ nanoparticles) (Haag et al, 2002) have been studied for the potential use of these carriers in drug and gene delivery. Successful encapsulation was observed in all cases by the PEI-based carriers while complexation was not observed with the PG-based carriers for the same guest molecules.

The objective to develop a pH-sensitive carrier was tested using several buffer solutions for both the acetal- and imine-bound shells. The encapsulated congo red in the...
carrier PGb was stable for several months at neutral and basic pH values (pH>7). However, an immediate release of the guest molecules occurred in acidic media (pH<3). The imine-based carriers were even more sensitive to an external decrease of pH. In the case of carrier PEIa (Table 5) the hydrolysis of the shell and the release of the encapsulated guest (namely, congo red) occurs over a period of four days at pH 6. However, it is stable over several weeks at neutral pH. On the other hand, the hydrolysis of the carrier PEIb and the release of the encapsulated guest occurs spontaneously even at pH<7. In the case of PEIb, a slow release can be observed after several hours (pH 8, ca. 3 h, 25 °C) or days (pH 12, 2 days, 25 °C) even without acidification. For the PEI-based carrier, PEIb, the amount of dye due to imine hydrolysis was followed by IR spectroscopy through the disappearance of the imine signal (Figure 20).

Figure 18. Boronated third generation PAMAM functionalized with PEG-Folate moiety.

Table 4. Biodistribution of non-pegylated (A), PEGylated (B) and PEGylated with attached folic acid (C) boronated poly(amidoamine) dendrimers.* Reproduced from Skukla et al, 2003 with kind permission from American Chemical Society.

<table>
<thead>
<tr>
<th>tissue</th>
<th>% ID of A/ g tissue</th>
<th>% ID of B/ g tissue</th>
<th>% ID of C/ g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>1.1±1.3</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>liver</td>
<td>20.6±5.0</td>
<td>7.1±4.0</td>
<td>38.5±5.9</td>
</tr>
<tr>
<td>spleen</td>
<td>14.7±3.9</td>
<td>20.2±8.8</td>
<td>25.0±7.4</td>
</tr>
<tr>
<td>kidney</td>
<td>104.2±20.7</td>
<td>45.0±13.4</td>
<td>62.8±14.5</td>
</tr>
<tr>
<td>muscle</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>tumor</td>
<td>n.m.</td>
<td>n.m.</td>
<td>6.0±1.6</td>
</tr>
</tbody>
</table>

* 24JK-FBP tumor bearing C57BL/6 mice were injected ip with A, B, and C. The % ID/g were determined 6h post injection. Mean ±SDs are based on four animals per group. n.m.: not measurable.

Figure 19. Functionalization of PG and PEI hyperbranched dendritic polymers. Reproduced from Krämer et al, 2002 with kind permission from the authors and Wiley-VCH.

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In another recent study, the same as above polyglycerol exhibiting low toxicity and biocompatibility, was functionalized for developing drug nanocarriers whose drug release can be salt-triggered. The utmost objective of this hyperbranched polymer functionalization is, as it is the case with dendrimers, to simultaneously address the main issues encountered with drugs themselves, as well as with their carriers, i.e., water solubility, stability in biological milieu and targeting.

In this context, PEGylated and PEGylated-Folate functional derivatives of polyglycerol, i.e. PG-PEG and multifunctional PG-PEG-Folate (Figure 21) were prepared and investigated as potential drug delivery systems (Tziveleka et al, 2006). For investigating this possibility, experiments have been performed employing PY and Tamoxifen (TAM), (Figure 22), an anti-cancer hydrophobic drug, for studying their encapsulation and release properties.

Table 5. Encapsulation capacities of congo red in dendritic nanocarriers based on PG and PEI. Reproduced from Krämer et al, 2002 with kind permission from Wiley-VCH.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mn core [gmol⁻¹]</th>
<th>Shell</th>
<th>Degree of alkylation</th>
<th>Encapsulation capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>21 000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PGa</td>
<td>21 000</td>
<td>H₃₁C₁₅</td>
<td>25%</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>PGb</td>
<td>21 000</td>
<td>H₃₃C₁₆O₆C₁₆H₃₃</td>
<td>45%</td>
<td>13±4</td>
</tr>
<tr>
<td>PGc</td>
<td>21 000</td>
<td>H₃₃C₁₆O₆C₁₆H₃₃</td>
<td>55%</td>
<td>2±0.5</td>
</tr>
<tr>
<td>PEI</td>
<td>25 000</td>
<td>-</td>
<td>-</td>
<td>0.02±0.005</td>
</tr>
<tr>
<td>PEIa</td>
<td>25 000</td>
<td>H₃₁C₁₅</td>
<td>33%</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>PEIb</td>
<td>25 000</td>
<td>H₁₁C₅C₅H₁₁</td>
<td>53%</td>
<td>0.2±0.05</td>
</tr>
</tbody>
</table>

Figure 20. Time-dependence of the shell cleavage of PEI-imine 4b on a KBr plate. Insert: The IR band of the imine peak at 1655 cm⁻¹ decreases because of cleavage, while the N-H out-of-plane vibration at 1565 cm⁻¹ increases. Reproduced from Krämer et al, 2002 with kind permission from the authors and Wiley-VCH.
Sideratou et al: Dendritic polymers for application as drug and gene delivery systems

![PG-PEG](image1)

**PG-PEG**

![PG-PEG-Folate](image2)

**PG-PEG-Folate**

**Figure 21.** Functionalized PG-PEG and PG-PEG-Folate hyperbranched dendritic polymers.

**Figure 22.** Chemical structure of Tamoxifen (TAM).

Based on promising results on pyrene encapsulation and release, the loading capacity and release properties of the polyglycerol derivatives for TAM were also investigated. TAM is a non-steroidal antiestrogen drug, which is widely used in the treatment and prevention of breast cancer (Wiseman, 1994; Mocanu and Harrison, 2004). Its encapsulation and release was comparatively investigated for the parent polyglycerol, PG, PG-PEG and the multifunctional PG-PEG-Folate derivative. The solubility of TAM in water was found to be $1.9 \times 10^{-6}$ M. Its solubility, however, increases by a factor of 5 when solubilized in PG solution (Table 6). The solubility of TAM is considerably further enhanced by a factor of 65 in the presence of PG-PEG. This significant solubility increase indicates that TAM is not only solubilized inside the hyperbranched interior but also inside the covalently bound poly(ethylene glycol) chains. This is in line with previous results employing PEGylated dendrimeric derivatives (Sideratou et al., 2001; Paleos et al., 2004) and other hydrophobic drugs, establishing that the introduction of the poly(ethylene glycol) chains in general enhances the solubilization efficiency of dendritic polymers. It is interesting to note that for PG-PEG-Folate a ~1300-fold increase of TAM solubility was observed.

Solubilized molecules can be replaced by the metal ion and it is, therefore, necessary to investigate whether sodium cation complexation can cause premature release of the drug in the extracellular fluids, before the nanocarrier loaded with the drug reaches the target cell. By titrating TAM loaded polymeric solutions with sodium chloride solution, the drug was released and suspended in the bulk aqueous phase. In the presence of 0.142 M NaCl solution, 39% and 24% of the solubilized TAM in PG and PG-PEG (Figure 23) were released respectively in the aqueous media. Under the same conditions and in the presence of PG-PEG-Folate, only 6% of the solubilized TAM was released (Figure 23). It should, therefore, be noted that for the most elaborated derivative prepared in this study, i.e. the multifunctional PG-PEG-Folate, most of TAM remains encapsulated in the polymer and it is not released in the extracellular fluid at a concentration of 0.142 M NaCl solution. Therefore, this nanocarrier can reach target cells appreciably loaded with TAM.

These results have to be taken into consideration before PEGylated polyglycerols are to be applied as drug delivery systems in experiments in vitro and in vivo. Sodium cation, in extracellular fluids can form complexes with PEG chains affecting the overall release profile of the drug. It is therefore required, for designed PEGylated drug delivery systems, to investigate whether drug release occurs in the extracellular fluid and before entering the target cells.
Table 6. Solubility of Tamoxifen in PG, PG-PEG and PG-PEG-Folate aqueous solutions. Reproduced from Tziveleka et al, 2006 with kind permission from Wiley-VCH.

<table>
<thead>
<tr>
<th>Hyperbranched Polymer</th>
<th>(C_{\text{Polymer}} [M])</th>
<th>(C_{\text{Tamoxifen}} [M])</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>(1.0 \times 10^{-3})</td>
<td>(9.6 \times 10^{-6})</td>
</tr>
<tr>
<td>PG-PEG</td>
<td>(1.0 \times 10^{-3})</td>
<td>(1.23 \times 10^{-4})</td>
</tr>
<tr>
<td>PG-PEG-Folate</td>
<td>(1.0 \times 10^{-3})</td>
<td>(2.48 \times 10^{-3})</td>
</tr>
</tbody>
</table>

Figure 23. Release of Tamoxifen from PG (●), PG-PEG (■) and PG-PEG-Folate (▲) aqueous solutions (1x \(10^{-3}\) M) as a function of added NaCl solutions. Reproduced from Tziveleka et al, 2006 with kind permission from Wiley-VCH.

IV. Gene carriers: from monofunctional dendrimers to multifunctional dendritic polymers

Numerous gene delivery systems based on viral (Verma and Somia, 1997; Lotze and Kost, 2002) and non-viral (Li and Huang, 2000; Brown et al, 2001; Nishikawa and Huang, 2001) vectors have been developed and tested so far. Recently, several recurring issues about safety of viral vectors have led to a careful reconsideration of their application in human clinical trials and prompted the use of synthetic systems. Moreover, viral vectors experience significant limitation in large-scale production and the available size of DNA they can carry. For addressing these problems, non-viral gene delivery systems such as cationic polymers or cationic lipids, liposomes or cationic dendrimers have attracted great attention for achieving a breakthrough in the development of an effective gene carrier. Specifically, synthetic non-viral carriers of genetic material present significant risks of genetic recombination in the genome. Transfection with synthetic vectors, through appropriate tailoring, may exhibit low cell toxicity, high reproducibility and ease of application. However, the currently known synthetic vectors present disadvantages, which are due to their generally low effectiveness compared to viral vectors and to their inability for targeted gene expression. For an effective gene expression, genes must be transferred in the interior of the nucleus of the cell and this procedure has to circumvent a series of endo- and exo-cell obstacles. Among these obstacles are included cell targeting, effective transport of the carriers together with attached genetic material through cell membranes and the need of carriers release from the endosome following endocytosis. For the synthetic carriers that have been described in the literature, some or all of these difficulties have been addressed, without, however, completely achieving this objective yet.

The strategy employed for the delivery of the conventional drugs through the preparation of functional dendritic polymers can also be applied for the delivery of genetic material. Specifically, the method involves molecular engineering of dendritic surface and/or the core aiming at obtaining polymers, which should be positively charged, biologically stable, non-toxic, exhibit targeting ability, and have the ability to be effectively transported through cell membranes. In addition, the dendrimer-DNA complex should have the possibility of being released from the endosome following endocytosis.

Dendrimers and hyperbranched polymers are stable nanoparticles in contrast to liposomes, which, as a rule, are unstable. Additionally, the dependence of dendrimers’ size on their generation can affect transfection efficiency. Several studies (Boas and Heegaard, 2004) have reported the use of unmodified amino-terminated PAMAM or DAB dendrimers as non-viral gene transfer agents, enhancing the transfection of DNA into the cell nucleus. The exact structure of these host–guest binding motifs has not been
determined in detail, but it is presumably based on acid-base interactions between the anionic phosphate moieties on the DNA backbone and the primary and tertiary amines of the dendrimers, which are positively charged under physiological conditions. It has also been found (Tang et al, 1996) that partially degraded (or fragmented) dendrimers, are more appropriate for gene delivery than the intact dendrimers and a fragmentation (or activation step) consisting of hydrolytic cleavage of the amide bonds is needed to enhance the transfection. These dendrimers are characterized as activated and are shown in Figure 24. It has been concluded from several investigations that the spherical shape of dendrimers is not advantageous in gene delivery. This agrees with earlier work, where ‘fragmented’ PAMAM dendrimers show superior transfection efficacy in comparison with the spherical ‘intact’ dendrimers (Boas and Heegaard, 2004).

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 (Dennig and Duncan, 2002). In addition, it is generally found that the maximum transfection efficiency (Figure 25) is obtained with an excess of primary amines to DNA phosphates, yielding a positive net charge of the complexes. The more flexible higher generation DAB dendrimers (containing no amides) are found to be too cytotoxic for use as non-viral gene vectors, however, the lower generation dendrimers are well-suited for gene delivery (Zinselmeyer et al, 2002).

Figure 24. Structural features of intact vs activated PAMAM Dendrimers.

Figure 25. Transfection efficacy of poly(propylene imine) dendrimers of various generations (G1-G5) relative to N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP) in the A431 cell line studied in 96-well plates. DAB G1 and DAB G2 were dosed at a dendrimer: DNA ratio of 5:1, and a DNA dose of 20 μg per well was used. DAB G3 was also dosed at a dendrimer: DNA ratio of 5:1, but a DNA dose of 5 μg per well DNA was used; DAB G4 and DAB G5 were dosed at a dendrimer: DNA ratio of 3:1 and a DNA dose of 20 μg per well. DOTAP was dosed at a DOTAP:DNA ratio of 5:1, and a DNA dose of 20 μg per well. Data represented as the mean ±SD of at least 3 replicates. Reproduced from Zinselmeyer et al., 2002 with kind permission from Springer.
In this connection, PAMAM-OH dendrimers, which are structurally similar to PAMAM, except that surface amino groups have been replaced by hydroxyl groups (Lee et al, 2003) have been prepared. Absence of surface primary amino groups in PAMAM-OH renders this polymer nearly neutral, which might be advantageous in terms of cytotoxicity. However, PAMAM-OH is nearly unable to form DNA polyplex because of the low pKa of interior tertiary amino groups (Tomalia et al, 1985). For this purpose, the synthesis and characterization of internally quaternized PAMAM-OH has been reported, as shown in the Figure 26. The internal quaternary ammonium groups of QPAMAM-OH will interact with negatively charged DNA, while preserving a neutral polymer and/or a polyplex surface.

It was found that QPAMAM-OH/DNA polyplexes were round-shaped with the more compact and small particles formed as the charge ratio increased. Although the transfection efficiency of functional QPAMAM-OH derivatives was lower by one order of magnitude than parent PAMAM (Figure 27), the QPAMAM-OH/DNA particles exhibited reduced cytotoxicity compared with PAMAM and PEI. Shielding of the interior positive charges by surface hydroxyl may be possibly the reason for this behaviour.

As already mentioned, one of the major problems with non-viral gene delivery systems is their lower efficiency compared to viral vectors. Many methods have tried to overcome such problems, including linking or conjugating cell-targeting ligands or cell penetrating peptides as efficient vectors for intracellular delivery of bioactive molecules (Futaki, 2005). Arginine-rich peptides have exhibited enhanced translocational ability, which was

![Figure 26. Quaternization of PAMAM-OH dendrimer.](image)

![Figure 27. Transfection efficiency of PEI, PAMAM and QPAMAM-OH dendrimers with 52, 78 and 97% quaternization degrees in 293T cell at charge ratio (+/-) = 6. Data are expressed as a RLU (Relative light unit) per µg protein. Reproduced from Lee et al., 2003 with kind permission from American Chemical Society.](image)
attributed to the presence of the guanidinium moiety (Vives et al, 1997; Rothbard et al, 2000; Wender et al, 2000; Futaki et al, 2001; Kirschberg et al, 2003), a structural feature of L-arginine, which is capable of hydrogen-bonding and electrostatic interactions (Onda et al, 1996) with phosphate or carboxylic group located at the surface of cell membranes. In a recent study (Choi et al., 1996) with phosphate or carboxylic group located at the dendritic gene vector are possibly satisfied in a PEI-oligonucleotides, and plasmids that are deficient in cell-incorporating small molecules, peptides, proteins, used as a dendritic nanocarrier encapsulating or relatively low cytotoxicity. These properties would make PEI(Lys) was prepared and tested showing slightly better transfection efficiency in HepG2 cells than that of basic PAMAM, while increased effect was not observed in primary cells. In conclusion, a polyvalent arginine functionalized PAMAM is easily prepared, which possesses outstanding transfection efficiency with relatively low cytotoxicity. These properties would make PAMAM-Arg a promising non-viral vector for both in vitro and in vivo use. Potentially, PAMAM-Arg could be used as a dendritic nanocarrier encapsulating or incorporating small molecules, peptides, proteins, oligonucleotides, and plasmids that are deficient in cell-penetrating.

The structural features set forth for a successful dendritic gene vector are possibly satisfied in a PEI-poly(ethylene glycol)-folate (PEI-PEG-FOL) derivative which was recently synthesized (Figure 30) and its efficiency as gene carrier was tested (Kim et al, 2005a). This multifunctionalization of PEI aimed at the preparation of a nanocarrier that would simultaneously combine protective and targeting properties. In this study, the PEI-PEG-FOL nanocarrier, was tested for its capacity to complex with plasmid DNA and be transfected to folate receptor overexpressing cells that produce exogenous green fluorescent protein, GFP, (GFP-KB cells). A special plasmid system (pSUPER-siGFP) was prepared, that carried a siRNA-expressing sequence, used for inhibiting the expression of exogenous GFP in mammalian cells. The pSUPER-siGFP/PEI-PEG-FOL complexes inhibited GFP expression of KB cells more effectively than pSUPER-siGFP/PEI (Figure 31). These results indicated that folate receptor-mediated endocytosis is a major pathway in the process of cellular uptake.

V. Concluding remarks

Molecular engineering of basic dendrimeric and hyperbranched polymer scaffolds resulted in the preparation of nanocarriers of low toxicity, with significant encapsulating capacity, specificity to certain biological cells and transport ability through their membranes. Depending on the degree and type of functionalization, products that fulfill one or more of the above characteristics were prepared. The exhibition of these properties is further induced by the so-called polyvalent interactions attributed to the placement of the functional groups in close proximity on the external surface of the dendritic polymers.

Figure 28. Introduction of L-Arginine at the external surface of PAMAM.

Figure 29
Figure 29. Transfection efficiency for Neuro 2A cell lines (1x10^5 cells/well). DNA amount per well was 0.2 µg (black) and 1.0 µg (gray). Values in parentheses are representing the charge ratio (N/P) of dendrimer/plasmid DNA complexes. The luciferase expression mediated by reagents was measured at each optimum condition. Results are expressed as mean ±SD of 3 replicates. Reproduced from Choi et al, 2004 with kind permission from Elsevier.

Figure 30. Chemical structure of PEI-PEG-FOL

Figure 31. GFP gene inhibition efficiency of pSUPER-siGFP/PEI and pSUPER-siGFP/PEI-PEG-FOL complexes as a function of N/P ratio against GFP-KB cells. Reproduced from Kim et al, 2005a with kind permission from Elsevier.
References


Constantinos M. Paleos