The role of macromolecular architecture in passively targeted polymeric carriers for drug and gene delivery

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(Received 16 November 2007; accepted 31 January 2008)

Abstract
The use of polymeric carriers for drug delivery has become increasingly popular because of the ability to easily tune the physical and biological properties of macromolecules. With the growing commercial accessibility of branched and dendritic polymers, their incorporation into polymeric carriers is being explored with increased frequency. However, while a handful of systematic studies have explored the use of branched macromolecules for drug delivery, the role of polymer architecture in optimizing the polymeric carriers is not yet fully understood. Herein, the authors summarize the extent that architecture has on the basic physical properties of polymers, and review our preliminary understanding of the architectural effects on polymer-assisted drug delivery.

Keywords: Polymer, dendrimer, macromolecular architecture, tumor targeting, gene delivery, drug delivery

Abbreviations: ATRP, atom transfer radical polymerization; CnVPP, poly(4-vinylpyridine) quaternized via a Cn alkyl chain; DNA, deoxyribonucleic acid; DOSPA, 2,3-dioleoyloxy-N-[2(3-sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate; DOTAP, N-[1(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or sulfate salts; DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride; HSA, human serum albumin; MRI, magnetic resonance imaging; NMP, nitrooxide mediated radical polymerization; PAMAM, poly(amidoamine); PCL, Polycaprolactone; PEG, poly(ethylene glycol); PEI, poly(etheramine); PHEMA, poly(hydroxyethyl methacrylate); PHPMA, poly(hydroxypropylmethacrylamide); PLL, poly(1-lysine); PPI, poly(propylene imine); PVP, poly(4-vinylpyridine); PS, poly(styrene); PSMA, poly(styrene-co-maleic anhydride); RAFT, reversible addition fragmentation chain transfer polymerization; ssDNA, single strand DNA

Introduction
The use of macromolecules as carriers for medical applications has been studied extensively in applications as varied as tumor targeting (Maeda et al. 2006), gene therapy (Putnam 2006), Magnetic resonance imaging contrast imaging (Wikstrom et al. 1989; Margerum et al. 1997; Kobayashi and Brechbiel 2003), in vivo oxygen sensing (Briñas et al. 2005), and topical antiviral formulations (Jiang et al. 2005). While the importance of carrier molecular weight (Seymour et al. 1995) and surface functionality has been elucidated in many of these applications, the specific role that molecular architecture plays in optimizing the delivery of polymer bound therapeutics appears to be significant, but is much less understood. There have only been a limited number of truly systematic studies designed to understand the role of architecture in drug targeting, largely as a result of the synthetic difficulty of preparing polymer libraries with contrasting architectures, but comparable functionality, composition, and molecular weight. Without a carefully selected set of carriers with analogous properties, but contrasting
architectures, it is difficult to draw meaningful conclusions as to the relationship between structure and biological properties.

However, recent advances in polymer chemistry, particularly the development of controlled radical polymerization techniques such as atom transfer radical polymerization (Matyjaszewski and Xia 2001), nitroxide-mediated polymerization (Hawker et al. 2001), and reversible addition fragmentation chain transfer polymerization (Moad et al. 2005), as well as the application of highly efficient “click” couplings (Kolb et al. 2001; Moses and Moorhouse 2007), enable the facile synthesis and assembly of modular libraries of carriers, and therefore offer promise in elucidating the role of architecture in drug delivery.

In light of the limited number of truly systematic studies published to date, this review cannot provide a definitive summary of the role of molecular architecture, but rather will review the critical parameters that can be controlled, and highlight the few but very promising architectural case studies that inspire further research in this important field. One of the significant challenges in this area of research is that truly meaningful data can only be obtained by coupling synthetic expertise with well-designed biosaying. This is more likely to happen as cross-disciplinary collaboration between synthetic chemists and experienced pharmaceutical scientists is both initiated and funded. This review limits itself to the investigation of covalently bound macromolecular architectures, though clinically meaningful work has been carried out with self-assembled systems, such as liposomes (Cvek et al. 1997; Touitou et al. 2000; El Maghraby et al. 2006; Elsayed et al. 2007), and micelles (Rangel-Yagui et al. 2005; Torchilin 2007), where the architecture cannot be controlled with the same degree of precision.

In the 1970s, two areas of polymer research explored the immense potential of macromolecules for drug delivery: their attachment to small drugs and their conjugation to proteins. Both techniques were developed in attempts to modulate the physical properties of the biologically active species. De Duve et al. (1974) first proposed that by boosting the molecular weight of small molecule drugs conjugated to polymers, the endocytic pathway of cellular uptake rather than random diffusion would be targeted. This, in turn, would favor the absorption of the therapeutic into the lysosomes, where pH changes could trigger drug release. As an extension of this concept, Ringsdorf (1975) proposed the bold goal of designing the ideal therapeutic by tailoring the critical properties of the macromolecular carriers: including molecular weight, drug loading, targeting moieties, and triggered drug release. Around the same time, the first conjugations of polymers to proteins were carried out. The initial work involved the attachment of poly(ethylene glycol) (PEG) chains to bovine serum albumin and bovine liver catalase (Abuchowski et al. 1977a,b). Not only did both proteins retain their activity but also demonstrated improved solubility, increased stability (by protecting the compound from enzymatic degradation), and reduced immunogenicity (by obscuring the surface of the proteins with non-immunogenic PEG). Since then, a range of techniques has been developed to attach PEG to improve the physical properties of a variety of therapeutics (Pasut and Veronese 2006). As a result, a number of PEGylated peptides (Veronese and Pasut 2005) and small molecule drugs (Pasut et al. 2004; Pasut and Veronese 2007) have been approved for commercial use to address numerous diseases.

From an early stage, it was apparent that the choice of polymer would be critical in determining its utility in bioconjugation. For example, while PEGylation became a popular method of increasing water solubility and reducing immunogenicity, the ability to functionalize only the two end groups of PEG led to unfavorably low loading efficiencies. In response to this, a number of linear polymer backbones with repeat units capable of functionalization have been explored for pharmaceutical use (Pasut and Veronese 2007), including poly(styrene-co-maleic anhydride) (Konno et al. 1984), poly(hydroxypropylmethacrylamide) (PHPMA; Lloyd et al. 1983; Kopcew et al. 2000), poly-L-glutamic acid (Singer et al. 2003), and dextran (Mehvar 2003).

In addition to optimizing loading, the biological interactions of the polymers in vivo become a critical issue. The functionalities along the polymer backbone are critical to determining whether the polymer will degrade readily under physiological conditions. Polysters, polyamides, and polysaccharides are popular biodegradable materials as the linkages between each monomer are readily susceptible to enzymatic or acid-catalyzed hydrolysis. For some applications where structural integrity is important, non-degradable but biocompatible polymers of the appropriate molecular weight are required. Poly(olefins) such as PHPMA and poly(hydroxyethyl methacrylate) (PHEMA) are ideal, as the uninterrupted carbon chain demonstrates long-term stability in vivo.

However, for non-degradable systems, the molecular weight of the polymer becomes a critical issue as polymers with too low of a molecular weight are rapidly removed from the bloodstream via glomerular filtration, while polymers with too high of molecular weight tend to accumulate in the liver and elsewhere (Seymour et al. 1995). The functional groups exposed to the external environment dictate both the solubility and the biocompatibility of a polymer. As a result, most polymers that bear water-soluble side chains, particularly alcohol functionalities, have seen the most use for macromolecular delivery scaffolds.

Until the last decade, polymer therapeutics focused almost exclusively on linear polymers, owing largely
to the difficulty in synthesizing more complex architectures with narrow dispersity on a commercial scale. Within the last two decades, advances in synthetic techniques have enabled the facile preparation of more complex architectures enabling researchers to explore how polymer architecture affects drug delivery. Before discussing the biomedical advantages of particular polymer architectures, the basic physical characteristics of different polymer architectures will be reviewed.

**Physical structure–properties relationships**

**Linear polymers**

Because the repeat units make up the vast majority of a linear polymer’s composition, its properties are generally dominated by the nature of the repeat unit and any side chain functionality that may be present, rather than the two end groups.

*Conformation in solution.* The conformational dynamics of a linear polymer in solution is affected primarily by two factors, the rotational freedom of the bonds along the polymer backbone, and the nature of the solvent. Because the majority of polymers of biological interest are dominated by sp³ hybridized atoms, they exhibit a wide range of conformations, depending upon the environmental conditions. When dissolved in a “good solvent” that is compatible with the repeat units, the polymer chain is equally compatible with the solvent as itself and tends to sample a variety of random coil conformations. This configuration resembles a loosely coiled ball and leads to moderate exposure of the repeat units to the solvent. In a “poor solvent,” the polymer is more compatible with itself than the solvent, and therefore will collapse into a much smaller globular configuration driven by the minimization of the surface energy. This phenomenon results in a compact conformation that minimizes the exposure of the repeat unit to the external environment. An important exception to this general view of polymer conformation can occur if the repeat units of the polymer exhibit a stereoregular pattern. Such polymers tend to be more crystalline if the surface energy or side chain steric effects encourage the formation of a more rigid helical configuration. This observation has been noted in both synthetic polymers like poly(propylene) and biopolymers like peptide alpha helices. The result is that linear polymers can exhibit a wide range of conformations depending upon their repeat unit functionality, their stereoregularity, and their interactions with their environment.

*Solubility.* Linear polymers exhibit significantly reduced solubility over their monomers because of the multiplicity of intermolecular interactions. In the solid state, the polymers will be bound to neighboring chains through a variety of non-covalent forces including hydrogen bonding, electrostatic attraction, or van der Waals forces. Because of the multiplicity of repeat units, the number of interactive forces per molecule is increased, and therefore more thermal energy is required to isolate a macromolecule from its neighbors. The result is that polymers exhibit a significantly reduced solubility that decreases with increasing molecular weight.

*Reactivity.* One of the consequences of the solution phase conformation of linear polymers is that any moieties attached to the polymer end groups or backbone will not be readily accessible for reaction, especially in collapsed random coil configurations. Because of the high probability that a portion of the polymer chain will block access to any particular repeat unit, the rate of reactions along the backbone of the polymer is drastically reduced. As a result, the modification of repeat units is typically slow and only favored for reactions with a substantial driving force.

*Rheology.* When bulk linear polymer is in the molten state, the individual polymer molecules are proposed to move in a snake-like reptation such that the overall sample resembles a can of worms (De Gennes 1971). The chain ends have more degrees of freedom and therefore define the mobility of the macromolecule, leading the polymer in its movement through neighboring molecules. However, as the molecular weight of the sample is increased, the chain entanglements between polymer chains increase, causing an increase in viscosity (Edwards 1967). The ramifications of polymer rheology for in vivo applications will be discussed later.

**Dendrimers and dendritic polymers**

Dendrimers are perfectly branched “tree-like” polymers (Figure 1) that are synthesized in a stepwise fashion to ensure both their monodispersity and their exact number of branching layers or “generations” (Fréchet and Tomalia 2001). The properties of dendritic polymers are dominated largely by the end groups, as they account for half of the repeat units in the structure and are located at the interface of the macromolecule and its external environment. Dendrimers of sufficiently high generation, typically above the third generation (G3), exhibit a number of properties that differentiate them from linear polymers and that result from their unique architecture. As a result, they have been investigated for numerous biological applications (Lee et al. 2005a; Ambade et al. 2006).

*Conformation in solution.* Due to the highly branched nature of dendrimers, they exhibit a globular structure that can be crudely approximated as a sphere. Unlike linear polymers, free rotations around atomic bonds do not lead to a significant change in molecular conformation, and therefore the molecules can be thought of as shape-persistent molecular spheres. This can be observed by comparing the retention time
of dendrimers relative to linear analogs using gel permeation chromatography. Dendrimers exhibited a distinct increase in retention time, correlating with a smaller hydrodynamic radius and a more compact conformation (Hawker and Piotti 2000). Imaging of dendrimers on solid surfaces has confirmed their spherical shape, though they have demonstrated the ability to flatten significantly if there are strong attractive forces to the surface (Hierlemann et al. 1998; Li et al. 2000). Much like linear polymers, dendrimers are expected to swell in good solvents and contract in bad solvents. However, the volume change is significantly less dramatic than in linear systems, as the highly branched architecture precludes a more extended “linear” conformation.

Early models of the dendritic conformation suggested that dendritic molecules would exhibit an extended conformation where the end groups would be located largely at the surface of the globular molecules (De Gennes and Hervet 1983). However, alternative models suggest that the end group could backfold to fill some of the void volume found near the core of dendritic molecules (Lescanec and Muthukumar 1990). Since these early models, numerous studies have been carried out to determine the position of the end groups, and it is generally accepted that backfolding does occur, but that the vast majority of the chain ends reside at the periphery of the molecule.

Solubility. Dendritic polymers exhibit a significantly enhanced solubility over linear polymers of analogous composition and molecular weight. This comparison has been demonstrated for both poly(phenylenes) (Miller et al. 1992) and poly(aryl esters) (Wooley et al. 1994; Hawker and Piotti 2000), and is largely a result of the globular conformation of dendrimers. The spherical shape affords the minimum surface area per volume ratio, and therefore a spherical molecule will experience fewer intermolecular interactions to lock it in the solid phase than would a linear polymer of comparable molecular weight.

Reactivity. Numerous reports have demonstrated that the end groups of dendrimers exhibit a significantly increased reactivity with respect to linear analogs (Wooley et al. 1994). For example, when comparing the hydrogenolysis of benzyl ether and ester end groups in both linear and dendritic poly(aryl esters), the linear systems were completely unreactive, while the dendritic systems exhibited a rapid and full deprotection (Wooley et al. 1994). There are many potential causes for this increased reactivity, and they depend largely on the type of reaction that is being measured. The improved solubility is clearly a significant factor in increased reactivity, as solution phase kinetics are typically orders of magnitude faster that solid phase kinetics. In addition, the accessibility of the chain ends is expected to be increased significantly. Because the majority of the end groups reside at the surface, they are more available for reaction. While substantial data suggest that end groups can backfold into the core of the dendrimer, the increased rate and yields for dendritic surface modifications indicate that any backfolded end groups exchange with the surface groups rapidly enough to ensure high accessibility.

Rheology. Studies in the rheology of dendrimers have clearly demonstrated that high degrees of branching have a profound effect on how these macromolecules interact with each other, and therefore also affect their bulk physical properties. For example, trends in the melt viscosity of dendrimers suggest that unlike linear polymers, their intermolecular interactions are not dominated by chain entanglement above a certain critical molecular weight (Hawker et al. 1995). Characterization of the intrinsic viscosities have verified that the viscosities of dendrimers increase with generation until passing through a maximum, and then decrease for larger generations (Mourey et al. 1992). These two pieces of data confirm early predictions (De Gennes and Hervet 1983) that the high degree of branching (DB) in the structure leads to a compact structure with a highly congested surface. The result is that at high generation number intermolecular chain entanglement is suppressed, which results in globular “molecular ball bearings” (Hawker et al. 1995).

Hyperbranched polymers

Hyperbranched polymers can be considered in both structure and properties as an intermediate between
strictly linear polymers and perfectly branched dendrimers (Figure 1). For hyperbranched polymers, the DB is a structural characteristic that determines the ratio of branching vs. linear monomers, and therefore defines where this molecule fits along this continuum. This measure was defined as the number of dendritic monomer units (connected to three other monomer units) + the number of terminal monomer units (connected to only one other monomer unit)/the total number of monomer units (linear + branched + terminal) (Hawker et al. 1991). Because dendrimers consist of only branched and terminal units, their DB will always equal one, while a long linear chain with only two terminal units would have a value close to zero. The DB for hyperbranched polymers will always fall between these two extremes and can be thought of as a crude measure of whether their properties will more closely resemble linear polymers or dendritic polymers (Table I). In addition, more detailed examination has demonstrated that the length of linear components in between branch points is another critical factor in predicting the physical properties of hyperbranched polymers (Markoski et al. 2001).

**Cyclic polymers**

Cyclic polymers represent an additional topological architecture beyond the more common linear and branched (Semyen 2000). The use of cyclic polymers for drug delivery has been largely overlooked due to the difficulty in preparing pure, low polydispersity cyclic polymers on a reasonable scale. Recent advances in synthetic techniques have enabled broader access to this unique topology, including ring-opening metathesis polymerization using a cyclic catalyst (Bielawski et al. 2002), ring-opening polymerization with a cyclic initiator (Culkin et al. 2007), and click cyclization of linear precursors (Laurent and Grayson 2006). The increased availability is expected to enable exploration of their applications.

**Conformation in solution.** The conformation of a cyclic polymer in solution is expected to follow the same trends as linear polymers, with the additional confinement that they have a circular rather than linear topology. In bad solvents, they are expected to collapse into a compact conformation, while in good solvents they should swell to a more extended conformation (Bensafi et al. 2000). However, the change in hydrodynamic volume is expected to be less than seen for linear polymers, since the linear polymer has end groups that can expand relatively independently of each other. This additional constraint in cyclic polymer has been observed as a subtle shift to a reduced hydrodynamic volume when comparing cyclic polymers with their linear analogs using gel permeation chromatography (Roovers and Toporowski 1983).

**Solubility and reactivity.** The reactivity and solubility of the repeat units of a cyclic polymer are not expected to be significantly different than those of a linear polymer, since the change in configuration is relatively minor. A few examples of backbone modifications on cyclic polymers have been reported (Schappacher et al. 1999; Laurent and Grayson manuscript in preparation) but kinetic data are too limited to draw significant conclusions. Likewise, detailed empirical studies on the effect of topology on solubility are needed to elucidate the physical properties of cyclic polymers.

**Rheology.** One of the more interesting effects of the cyclic architecture relates to its rheology. In linear polymers, the rheology is strongly affected by the end groups, as they are the least constrained by their neighboring repeat units. Cyclic polymers that lack end groups are expected to behave quite differently both in bulk and in solution. The generally accepted model is that their motion will resemble that of an ameba, where portions of the loop can thrust out or contract within the confines of neighboring molecules (McLeish 2002).

**Table I. General trends in the properties of polymers with respect to the DB.**

<table>
<thead>
<tr>
<th>DB</th>
<th>Linear</th>
<th>Hyperbranched</th>
<th>Dendritic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>Conformation in good solvent</td>
<td>Extended</td>
<td>Intermediate</td>
<td>Globular</td>
</tr>
<tr>
<td>End group reactivity</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
</tr>
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of polymerization of the backbone, molecular weights ranging from tens to thousands of kilodaltons can be easily accessed (Lee et al. 2005b) and a wide range of polymer backbones can be incorporated (Figure 2), including poly(styrene) (PS; Grayson and Fréchet 2001), poly(propylene) (PCL; Lee et al. 2004), poly(l-lysine) (PLL; Lee and Fréchet 2006), PHEMA (Nyström et al. 2005), and carbonane-containing acrylate monomers (Benhabbour et al. 2007).

Biological structure–properties relationships

Biocompatibility

The biocompatibility of a polymer is dependent primarily upon the identity of those functional groups sufficiently exposed to interact with biological tissues in vivo. While polymer architecture alone does not determine biocompatibility, the structural persistence of highly branched molecules can be used to modulate biocompatibility. Because branched architectures can reduce access to the core of a macromolecule, enzymatically unstable or non-biocompatible functionalities can be encapsulated within a branched polymer to minimize undesired interactions in vivo. A number of polymer backbones, including PEG, PHPMA, SMANCS, and poly(glutamate) are sufficiently biocompatible to have been used in clinical studies, and the results of these studies are reviewed elsewhere (Satchi-Fainaro et al. 2005; Vicent and Duncan 2006).

PEGylation is a well-known technique for improving the water solubility and biocompatibility of materials while minimizing non-specific protein absorption or cell adhesion. PEG conjugation has been used widely to improve the biological utility of small molecules, proteins, polymers, surfaces, and even artificial organ implants. For example, linear PEG grafts have been used to increase the water solubility and reduce the cytotoxicity of a range of linear polymers, including PLL (Sawhney and Hubbell 1992; Bogdanov et al. 1996; Banaszczuk et al. 1999), poly(cyclooctene) (Breitenkamp et al. 2002), PCL (Parrish et al. 2005; Rieger et al. 2006), chitosan (Mao et al. 2005; Huang et al. 2006; Liu et al. 2006), and poly-(N-isopropylacrylamide-co-maleic anhydride) (Koseli et al. 2003). However, PEGylation is particularly valuable when a required component of a drug delivery system also demonstrates poor biocompatibility. For example, polyamines are vital for the complexation of deoxyribonucleic acid (DNA) during gene delivery but exhibit high cytotoxicity and hemolysis because of the formation of polycations at physiological pH. The addition of PEG grafts effectively attenuates the cytotoxicity of branched poly(ethyleneimine) (PEI) and correlates directly with the weight percentage of PEG grafts (Sung et al. 2003). However, the structural distribution of the copolymer was equally significant in determining the biocompatibility. For copolymers with nearly identical weight ratios of branched PEI and PEG, copolymers with more numerous, but shorter PEG chains distributed throughout the structure improved the biocompatibility better than a single larger PEG chain (Figure 3; Peterson et al. 2002b,c). This reinforces the theory that the biocompatibility of the most accessible functional groups in vitro is the critical factor in determining the overall biocompatibility of a polymer. However, other factors might play a significant part in the reduced cytotoxicity of the highly PEGylated PEI copolymers, such as the reduced number of primary amines or the larger weight percentage of linkers.

Numerous dendrimer end-group modification studies add further evidence to the theory that the exposed functionalities are the primary factors in determining the biocompatibility of a macromolecule. For example, while amino-terminated poly(amideamine) (PAMAM) dendrimers are natively quite hemolytic and cytotoxic (Malik et al. 2000), those with carboxylate end groups demonstrate significantly improved biocompatibility (Wiwattanapatapee et al. 2000). In addition, the biocompatibility of PAMAM dendrimers can be improved by conjugating compatible end groups (Figure 4), such as PEG (Luo et al. 2002; Jevprasesphant et al. 2003b), arginine (Choi et al. 2004; Kim et al. 2006), dodecyl (Jevprasesphant et al. 2003a,b), and acetyl (Kolhatkar et al. 2007) end groups. Similarly, the biocompatibility of amine-terminated PPI dendrimers has been improved by end group functionalization with saccharides or amino acids (Agashe et al. 2006; Figure 5). While the reduced cytotoxicity is due, in part, to the conversion of the primary amine functional groups to amide...
functional groups, the biocompatibility of the highly PEGylated dendrimers more closely resembles PEG than that of amide-terminated dendrimers. Modification of melamine dendrimer surface functionalities to a variety of other moieties, either cationic, neutral, or anionic (Figure 6), demonstrates that the cationic surfaces generally produce higher cytotoxicity than their neutral or anionic counterparts (Chen et al. 2004). However, while anionic end groups such as carboxylates, phosphates, and sulfonates markedly improved the biocompatibility of amino-terminated dendrimers, PEGylation of the dendrimer end groups consistently yielded the most compatible macromolecules (Chen et al. 2004).

The reduced flexibility of highly branched structures also appears to have an effect on the biocompatibility. When comparing modestly branched PEI and highly branched amino-functionalized dendrimers, it has been observed that the more rigid globular systems demonstrated improved biocompatibility (Malik et al. 2000; Fischer et al. 2003). A similar trend has been observed in PAMAM dendrimers, where the cytotoxicity is slightly reduced with each successive generation (Malik et al. 2000). In addition, star PHPMA with conjugated doxorubicin demonstrated reduced cytotoxicity compared with linear models (Wang et al. 2000). For more highly branched systems, this might be explained by the fact that the core is less accessible due to reduced conformational flexibility, and therefore the cytotoxicity becomes dominated by the end groups (Duncan and Izzo 2005). However, some reports suggest that PAMAM dendrimers are more cytotoxic than their linear analogs (Richardson et al. 1999), and early in vivo toxicity studies suggested that higher generation amino-functionalized dendrimers were more toxic than their smaller counterparts (Roberts et al. 1996). These conflicting data confirm that additional research is required to ascertain the full effect of polymer architecture on cytotoxicity.

Aliphatic polyester dendrimers based on bis-(hydroxymethyl)propanoic acid exhibit a highly biocompatible structure that offers an attractive alternative to the PAMAM dendrimers for many drug delivery
applications (Figure 7). These hydroxyl-terminated dendrimers have recently become commercially available and exhibit water solubility and low cytotoxicity (Padilla De Jesus et al. 2002). The versatility of the divergent preparations of these dendrimers enables their incorporation into a variety of hybrid structures, such as dendrimer-core star polymers, dendronized star polymers (Figure 8; Ihre et al. 2002), and dendronized linear polymers (Figure 9; Lee et al. 2004). Because of the inherent biocompatibility of the hydroxyl surface, additional modification is not necessary. In addition, like polyethylene glycol, grafting these dendritic side chains appears to attenuate the cytotoxicity of otherwise incompatible parent polymers (Lee et al. 2005b).

Because of their conformational persistence, dendrimers and highly branched polymers provide an attractive method for isolating components of medical interest from their in vivo biological environment. If the branched polymer has the appropriate rate of biodegradability or of host release, the encapsulated compound can be liberated after the carrier has localized to the intended target site. This technique of masking is attractive for a range of applications, including the attenuation of the toxicity of cancer drugs during tumor targeting and the protection of DNA plasmids or siRNA from enzymatic degradation during gene delivery. Making use of this unique dendritic host approach, recent research has involved the encapsulation and delivery of quinoline-based
drugs (Cheng et al. 2007), carboranes for boron neutron capture therapy (Benhabbour et al. 2007), and gold nanoparticles for photothermal therapy (Haba et al. 2007; Shi et al. 2007).

Biodegradability

The biodegradability of polymers is a critical issue for most medical applications. While high molecular weight polymers offer a number of attractive properties, such as protection of guest molecules from enzymatic degradation, increased blood circulation time, and favorable accumulation in tumor tissue through the enhanced permeability and retention (EPR) effect; molecules larger than 40 kDa cannot be cleared efficiently, resulting in accumulation in the body and undesirable side effects. The ability to incorporate biodegradable linkages into the polymer scaffold therefore becomes vital to enable the breakdown and eventual clearance of the polymeric carriers.

For linear polymers, a number of chemical linkages have been employed in the synthesis of biodegradable materials and are reviewed elsewhere (Nair and Laurencin 2006). Natural polymers are a logical choice for biodegradable materials, and research has focused chiefly on polysaccharides and polypeptides. The natural abundance, efficient enzymatic degradation, ease of functionalization, and relatively low immunogenicity of carbohydrates make polysaccharides a seemingly ideal scaffold for drug delivery. Numerous polysaccharides have been employed for drug delivery, including cellulose, amylase (starch), dextran, hyaluronic acid, chitin, and chitosan (Figure 10).

Most commercial synthetic polymers are based on olefinic, styrenic, acrylate, or methacrylate monomers and demonstrate negligible rates of degradation owing to the chemically hearty carbon–carbon bonds along the length of their backbone. For this reason, the use of such polymers with molecular weights above 40 kDa is significantly limited in the body.

Synthetic polyesters offer the desired balance of stability for short time periods, yet degradability over extended periods to small molecule components via enzymatic or hydrolytic cleavage. Ring-opening polymerization methods have been developed for the
synthesis of a number of polyesters, such as poly(glycolic acid), poly(lactic acid), and poly(caprolactone) (Figure 10), and their use for medical materials is particularly widespread due to their ease of synthesis and their narrow polydispersity. Other synthetic polymers that have been investigated for biodegradable materials include poly(orthoesters), poly(amide)s, poly(phosphazenes), and poly(phosphoesters).

While polyethylene glycol and PAMAM dendrimers are two of the more common polymer components in medical studies as a result of their well-defined molecular weight distributions and their commercial availabilities, neither demonstrates a favorable rate of degradation. Due to the chemically stable aliphatic ether linkages, the enzymatic degradation of PEG can occur only slowly via alcohol dehydrogenase (Kawal 2002) and P450 (Friman et al. 1993). Likewise, PAMAM dendrimers are known to be susceptible to amide hydrolysis (Tang et al. 1996) and bond cleavage via retro-Michael additions (Peterson et al. 2002a), but these reactions are significantly slower under physiological conditions than the rate of ester hydrolysis.

A number of synthetic polyester dendrimers offer the combination of both branching and biodegradability. Using components that are common biological
metabolites, a rather elegant biodegradable and biocompatible polymer has been constructed of glycerol and succinic acid (Figure 7; Carnahan and Grinstaff 2001). In addition to regenerating these innocuous small molecules upon degradation, the rates of degradation can be finely tuned by varying the nature of the diacid component. For example, dendrimers of lactic acid and glycerol degraded much more rapidly, owing the favored backbiting reaction to form a δ-lactone (Grinstaff 2002). Another attractive family of dendritic polyester is prepared from the bis(hydroxymethyl)propanoic acid repeat unit (Ihre et al. 1996, 1998, 2001, 2002). The pivalate esters of the backbone are more hydrolytically stable and demonstrate long-term stability in mildly acidic conditions (Lee et al. 2004), owing to the steric hindrance around the electrophilic carbonyl, yet exhibit a slow degradation under mildly basic conditions and rapid degradation in strongly basic solutions (Grayson and Fréchet 2002).

For enabling a triggered and accelerated degradation, a particularly elegant approach has been developed recently whereby the branching in dendritic architectures can be designed to enable a cascade of bond cleavages (Amir et al. 2003; De Groot et al. 2003; Li et al. 2003). These “self-immolative” dendrimers take advantage of the fact that the generation of the phenolate anion can lead to the rapid scission of any ortho or para benzyl ether linkages (Figure 12). This is a particularly attractive approach for drug release as different drug groups can be released simultaneously to enable synergistic therapies. A variety of reactions can be used to initiate the domino effect of bond scission, including photolytic or enzymatic cleavage of the trigger bond.

The construction of hybrid architectures out of linear and dendritic components offers an efficient route to prepare highly branched, high molecular weight polymers that will degrade into components that will be cleared through glomerular filtration. For example, the attachment of linear PEG chains to a small dendritic core via cleavable ester bonds provides a carrier with a molecular size (>40 kDa) large enough to afford a long lifetime in the bloodstream and tumor targeting via the EPR effect, yet will breakdown into readily cleared PEG chains (<20 kDa) and other small molecules (Gillies et al. 2005). Likewise, dendronized linear polymers offer an efficient route to highly branched molecules with molecular weights in excess of 1000 kDa, which can be readily degraded to enable long-term clearance (Lee et al. 2005b).
Figure 8. Dendronized star polymer-based three-armed star PEG core and G3 aliphatic polyester dendrons.

Figure 9. Dendronized linear polymer consisting of aliphatic polyester dendrons on a poly(caprolactone) backbone.
Blood circulation time

Most polymer–drug conjugates are administered intravenously or orally and depend upon the circulatory system to carry the therapeutic to the desired site of treatment. However, the lifetime of a molecule in the circulatory system is limited by the efficient filtration of foreign materials and waste by the kidneys. These materials are then excreted from the body in the urine. In order to ensure that the lifetime of the therapeutic in the circulatory system is sufficient to enable successful targeting and activity, the molecules must be optimized to reduce first-pass metabolism.

Empirically, linear polymers with high molecular weights and neutral or negatively charged surfaces exhibit sufficient blood circulation times to enable targeting (Sezaki et al. 1989). For example, dextrans with cationic side chains were shown to clear rapidly from the bloodstream independent of their molecular weight, while the same structures with anionic side chains demonstrated a much slower plasma clearance with increased circulation times for the larger polymers (Takakura et al. 1987). Similar results were observed with the chain end modifications of PAMAM dendrimers. Cationic amino-functionalized PAMAMs exhibited a rapid clearance from the bloodstream, while those with carboxylate end groups demonstrated a significantly increased blood circulation (Malik et al. 2000). Neutralization of the amine end groups by acetylation or PEGylation also increased circulation times compared with the parent amino-functionalized dendrimers, and reduced the undesired accumulation in other organs (Margerum et al. 1997). However, because of the tedious stepwise synthesis of dendrimers, it is difficult to efficiently prepare dendrimers of sufficient size to provide ideal circulation times. For example, the small size and compact nature of the G4 aliphatic polyester dendrimers lead to an insufficient half-life of less than 1 h in the bloodstream (Padilla De Jesus et al. 2002). Because of this synthetic limitation, the assembly of hybrid dendritic architectures is an attractive alternative that can boost the macromolecular size, while minimizing the synthetic effort (Margerum et al. 1997).

For the polyester dendrimers, the synthesis of three linear-dendritic hybrid architectures have been investigated to optimize the blood circulation times of branched macromolecules: dendrimer-core star polymers (Ihre et al. 2002), dendronized star polymers (Ihre et al. 2002), and dendronized linear polymers (Lee et al. 2005b). All three of these approaches have demonstrated a significant improvement in both synthetic accessibility and blood circulation times. Most notable, the dendronized linear polymers could be prepared rapidly with molecular weights ranging from 67 to 1700 kDa and blood circulation half-lives ranging from 14 to 44 h (Lee et al. 2005b).
Exploration of a linear-dendritic hybrid "bow-tie" system revealed the important role that branching plays in increasing the blood circulation time. Because of the modularity in the synthesis of these macromolecules, a unique set of bow-tie dendrimers were made with nearly identical molecular weights and hydrodynamic volumes but contrasting degrees of branching (Figure 13). These syntheses were carried out by attaching either two PEG chains of ∼20 kDa molecular weight, four PEG chains of ∼10 kDa molecular weight, or eight PEG chains of ∼5 kDa molecular weight to the dendrimer core to yield three drug carriers, each with a molecular weight of ∼45 kDa (Gillies et al. 2005). Comparison of the blood circulation half-lives of the three hybrids demonstrated a significant difference in blood circulation times from less than 1.4 h for the carrier with two 20 kDa PEG chains, to 31 h, for the carrier with eight 5 kDa PEG chains. It has been hypothesized that the cause of this effect is related to the deformation of these molecules in solution. The least branched structure will more closely resemble a linear polymer that can reptate through a narrow pore. On the other hand, the more branched structures, while expected to have a reduced hydrodynamic volume, will also have a more compact, rigid, globular configuration, and therefore cannot as easily diffuse through the same narrow pore as easily (Bohrer et al. 1979).

Molecular architecture appears to offer a parameter complimentary to molecular weight in the
optimization of the blood circulation time of polymeric carriers. Because the role of branching in slowing renal filtration has not been thoroughly explored, there are insufficient data from which to draw broad conclusions. However, additional studies involving well-designed libraries of macromolecules should further elucidate the advantages of branched molecules during the process of renal filtration.

**Biodistribution**

The fate of a drug conjugate in vivo is related largely to its size and exposed functionalities. Ideally, a carrier would be designed to have minimal nonspecific interactions and accumulate based solely upon the desired passive and active targeting mechanisms. Typically, however, the optimization of molecular weight or surface functionality for a specific target will also affect its interactions with sites throughout the body, and therefore it is impossible to develop a system that will localize exclusively within a specific tumor, cell-type, or organ. Instead, carriers must be optimized to maximize their accumulation at the desired site, while minimizing toxic effects elsewhere.

Whether actively or passively targeted, the first critical factor in controlling biodistribution is increasing blood circulation time. This will ensure that a macromolecular drug conjugate is in the bloodstream long enough to interact with and target the desired site of delivery, minimizing the percentage excreted from the body. However, while increasing the molecular weight is a critical factor in prolonging circulation time, it can also lead to increased exposure of the macromolecule to all organs (Veronese et al. 2005). For linear PHPMA, while the accumulation in organs was low relative to that in the tumor, higher molecular weight polymers demonstrated higher levels of accumulation in skin, liver, spleen, and muscle tissues (Seymour et al. 1995).

Early in vivo studies with tumor-free mice have demonstrated that amino-functionalized PAMAM dendrimers also have a tendency to accumulate in the liver, kidney, spleen, and pancreas, with the lowest concentrations observed for the higher generation (G7) dendrimers (Roberts et al. 1996). However, the addition of PEG chains onto the periphery of the PAMAM dendrimers appears to have effectively reduced liver retention while boosting half-life in the bloodstream (Margerum et al. 1997). For the poly(ester)-based dendronized polymers, extremely high molecular weight (>1000 kDa), macromolecules could be prepared, and initial biodistribution studies showed very high accumulation in the liver and spleen. While this observation was believed to have resulted from the drastically increased size of the molecules, the limited amount of data at present prevents unambiguous explanation (Lee et al. 2005b).

Owing to the fact that most therapeutics have a negative effect on healthy tissues, the preferential delivery of drugs to the desired site is a critical factor for an effective macromolecular therapeutic. For specific targeting in the body, the functionality and molecular weight of a macromolecular carrier must be tuned to enable sufficient exposure to the desired site while minimizing undesired accumulation in other portions of the body. The architectural effects for
optimal tumor targeting and gene delivery will be discussed in the following two chapters.

**Tailored polymer architectures for improved EPR targeting**

Conventional chemotherapy using small molecular weight drugs distributes the toxic therapeutic indiscriminately into both healthy and cancerous tissues (Maeda et al. 2006). As a result, these untargeted cancer treatments cause a range of hazardous side effects in the circulatory system, including anemia, thrombocytopenia, and neutropenia, resulting in suppression of the patient's immune system. In addition, drug accumulation in other tissues can cause cardiotoxicity and hepatotoxicity, which, when coupled with immunosuppression, can lead to a range of debilitating side effects and even death. However, new techniques for delivering drugs more specifically to cancer cells offer the promise of reducing the severity of chemotherapy side effects.

Two modes of targeting polymer therapeutics have been researched to focus the accumulation of toxic drugs more preferentially toward tumor tissues: active and passive targeting. Active targeting of polymer therapeutics involves the attachment of receptors, including folate, saccharides, or proteins causing the drug carrier to bind strongly to the surface of the cancer cells and encouraging their uptake into the desired cells. Passive targeting takes advantage of the innate physical properties of a polymer to enable preferential accumulation in a given tissue. The most effective method of passive tumor targeting, the EPR effect, uses the size and shape of a polymeric drug carrier to deliver...
the therapeutic into tumor masses (Matsumura and Maeda 1986, Duncan and Sat 2004). The critical factor that enables the effective targeting of tumor tissue is the unique permeability of the tumor vasculature. When tumor masses grow to a size of \( \sim 2-3 \) mm, neovascularization develops to supply the nutrient and oxygen demands for the rapidly growing tumor tissue (Folkman 1995). It has been demonstrated that the construction of this tumor vasculature differs significantly from healthy vasculature. Specifically, the endothelial cell lining contains much larger intercellular gaps than those typically found in healthy vasculature, and the outer smooth muscle layer is often deficient (Skinner et al. 1990), leading to a much "leakier" conduit. Because the endothelial cell spacing in tumor tissue is much wider, macromolecules that would be largely excluded from healthy tissue can diffuse with relative ease into tumor tissue. In addition, the lymphatic clearance within tumor tissues is reduced, leading to a preferential accumulation of macromolecules in tumor masses. While the fenestrated capillaries of the liver, spleen, bone marrow, and kidneys may also lead to some accumulation of macromolecules in these tissues (Simionescu 1983), the overall distribution of macromolecules to tumor tissues can be greatly improved over untargeted delivery (Sezaki et al. 1989; Maeda et al. 2006). The EPR effect offers a synergistic advantage: in addition to the increased percentage of drug dosage that accumulates at the tumor site, the reduction of deleterious side effects from untargeted drug delivery enable dosages to be increased even further.

In order to optimize a targeted polymeric carrier using the EPR effect, the size and the structure of the polymer must be carefully tuned. If the structures are too small, they will be rapidly cleared by the kidneys, but if they are too large (>40 kDa for linear systems), they can accumulate in the liver, spleen, and other organs (Vicent and Duncan 2006). Researchers have investigated the optimal size for linear polymer carriers by tuning the molecular weight of PEG (Yamaoka et al. 1994) and PHPMA (Seymour et al. 1995). However, as the actual theory for discrimination between tumor and healthy vasculature involves the preferential diffusion through the endothelial membrane, it is expected that size and conformational flexibility, and not molecular weight, are the critical factors in optimizing selective delivery.

Coiled polymers are believed to exhibit reduced permeation through porous membranes due to the larger girth of the polymer when repressing in solution (Uzgiris 2004; Uzgiris et al. 2004). Similar to the argument for increasing the blood circulation time of polymers (Bohrer et al. 1979; Gillies et al. 2005), the larger shape and reduced flexibility of a coiled—or branched—macromolecule are expected to increase the selectivity of the EPR effect. In other words, the enlarged girth of these molecules is predicted to increase their diffusion through the leaky vasculature of tumor tissue, relative to the tight endothelial gaps found in healthy vasculature.

Initial explorations of non-linear polymer architectures for tumor targeting have included the use of dendrimers, star—dendrimer hybrids, dendronized polymers, and linear-dendritic hybrid "bow ties." One of the earliest explorations of dendritic carriers involved the conjugation of cisplatin with PAMAM dendrimers, and demonstrated a promising 10-fold improvement in solubility as well as a five-fold increase in tumor targeting over free drug (Malik et al. 1999), although the cross-linking of these conjugates into larger aggregates is believed to affect their pharmacokinetics. Further optimization of the dendritic carrier concept involved the attachment of folate groups to a PAMAM—methotrexate conjugate, and demonstrated an additional 10-fold increase in tumor targeting (Kukowska-Latallo et al. 2005). While these observations highlight the value of both branched architectures and receptor-mediated binding, the typically small size of dendrimers in solution can result in rapid clearance from the blood stream with subsequent excretion of the majority of the polymer-bound therapeutic.

In response to the size limitation associated with traditional dendrimers, alternate dendronized architectures with vastly increased sizes have been investigated to further explore how the inclusion of branching can optimize the EPR effect. Dendronized linear polymers were prepared with a linear poly-(hydroxystyrene) core and fourth-generation polyester dendrons attached to each repeat unit, with molecular weights as high as 1700 kDa. Biodistribution studies showed that the tumor targeting for these dendronized linear polymers with an in vivo C-26 colon carcinoma model was significantly enhanced over other architectures investigated (Lee et al. 2005b), and comparable to the best results reported for this model using liposome-based carriers (Huang et al. 1992). Equally promising, the attachment of 16 doxorubicin drug groups onto PEG-bow tie dendrimers (Figure 14) afforded a macromolecular therapeutic that, in a single dose, cured 10/10 mice studied (Gillies and Frechet 2002, Lee et al. 2006). Prior to this investigation, the most successful therapeutic for this model was a clinically approved liposome-based doxorubicin delivery system (DOXIL; Larwood and Szoka 1984; Seymour et al. 1994) that required multiple doses to achieve the same effect.

It is clear that manipulation of polymer architecture, as well as size and functionality, can lead to vastly improved materials for EPR targeting of cancer cells. Highly branched polymer therapeutics demonstrate improved solubility, reduced toxicity, increased blood circulation times, and enhanced delivery to tumor tissue, leading to vastly improved therapeutics compared with linear analogs. While initial studies
have been promising, the limited number of systematic studies comparing the effects of size, shape, and branching has prevented full understanding of the relative importance of each factor. Additional research in this field will undoubtedly answer many of these questions and further optimize the passive targeting of tumors.

**Tailored polymer architectures for improved gene delivery**

**Polycations**

A commonality among most molecules used for nonviral gene delivery vehicles is a positive charge. Typically, this charge is carried by amine nitrogens and serves to form an electrostatic bond with polynucleotides that happen to be polyanions. Examples of polycationic carriers include PEI, PLL, PAMAM dendrimers, and chitosan. Gene delivery micelles also incorporate cationic entities in lipids such as 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride or sulfate salts.

In addition to presenting a favorable condition for electrostatic complexation with and condensation of polynucleotides, another benefit of using gene delivery vehicles with cationic moieties is the facilitation of cellular entry. Mammalian cells typically have a net negative charge on the extracellular face of the plasma membrane, and this condition makes attachment of gene delivery complexes with positive zeta potentials energetically favorable. For the polycation PLL, cellular entry of PLL–DNA complexes is accomplished by the complexes first binding to sulfated, membrane-associated proteoglycans (Wojda et al. 1999). The variable expression of proteoglycans on cell surfaces might help to explain the variation of transfection efficiencies between cell types. PAMAM–DNA complexes have been observed to enter endothelial cells via association with membrane cholesterol (Manunta et al. 2004). PEI–DNA complexes are internalized by a clathrin-dependent pathway in lung endothelial cells, regardless of the polarization state of the cells (Mennesson et al. 2005). Histidinylated PEI–DNA complexes enter unpolarized lung endothelial cells in a similar manner, but with an involvement of cholesterol (Mennesson et al. 2005). Cell binding studies have revealed that linear PEI, branched PEI, and PAMAM dendrimers (generations 2 and 4) have cell binding properties similar to each other at 37° (Seib et al. 2007). Interestingly, this same study demonstrated that the cell binding properties for each of these gene carriers is very poor at 4°, with the exception of branched PEI. Pinocytosis rate curves were also similar for each of the carriers, again with the exception of branched PEI, for

![Figure 14](image-url)

(PEO<sub>35</sub>)<sub>2</sub>–[G3]–[G4]–DOX<sub>16</sub> doxorubicin conjugate for tumor targeting study. A single dose provided an effective cure to implanted colon carcinomas in murine studies.
which a significantly lower percentage of cell-bound polymer was endocytosed (Seib et al. 2007). These results indicate that there is more than one mechanism for cellular binding and entry available to polycations, with branched PEI appearing to be relatively unique with regard to cellular interactions.

Following cellular entry, endocytosed (or pinocytosed) gene delivery complexes often are exposed to degradative enzymes such as nucleases found in (endo)lysosomes. Another benefit of using cationic gene delivery agents with high charge densities lies in the condensation of the carried DNA. This condensation can serve to protect the genetic material being delivered, which has been shown to be the case for many polymeric carriers. PEI has been shown to protect complexed DNA from DNase I and II digestion in a manner that is superior to protection offered by PLL (Godbey et al. 2000). The protection afforded by PEI was found to be due mainly to a physical barrier, as opposed to increasing local pH values to inactivate the pH-dependent DNase II. Extended digestion times with excess amounts of DNase I (over 5000-fold) were not sufficient to degrade PEI-protected plasmids. PAMAM dendrimers also afford protection against DNase I digestion, and this protection has been shown to be superior to that of liposomal packaging (Ernst et al. 1999). It has been observed during such degradation experiments that PAMAM (G4)—DNA complexes are dynamic, with individual dendrimer molecules being continually gained and lost by individual dendrimer—DNA complexes (Abdelhady et al. 2003).

At this point, it would seem that a higher cationic charge density would provide for better DNA encapsulation and stronger binding of complexes to cell membrane components, and thereby yield higher transfection efficiencies. This approach is not optimal for transfection, however, because many polycations are associated with cytotoxicity. For several polymer concentrations and cell types, a relative ranking of cytotoxicity has been found as follows: PEI > PLL > poly (dialyl dimethyl ammonium chloride) > diethylaminoethyl dextran > poly (vinyl pyridinium bromide) > PAMAM dendrimer > cationized albumin > native albumen (Fischer et al. 2003). There was a dependence of observed cytotoxic effects upon concentration and time. Again, cationic charge densities (as well as molecular weights) were found to be related to interactions with cell membranes, and such interactions led to cell damage.

While the number and density of cations is important to the potential transfection efficiency and cytotoxicity of a gene delivery vehicle, the architecture of the molecule also plays a key role. Differences in the cellular binding and entry rates of linear vs. branched PEIs have already been mentioned (Seib et al. 2007). In terms of DNA condensation, the two forms of the polymer also differ, with branched PEIs producing greater DNA condensation than the linear form (Dunlap et al. 1997). Also apart from cellular entry rates, architecture has an effect on the effectiveness of delivered genetic material. As an example, two sixth-generation PAMAM dendrimers—one intact and one fractured—have been used to deliver siRNA into a human epidermoid carcinoma cell line (Hollins et al. 2007). While the cells took up each type of gene delivery complex at a similar rate, transfections with the intact dendrimers yielded a greater interference effect. Use of the fractured dendrimers at low (10 nM) concentrations actually stimulated expression of the targeted gene vs. negative controls. Higher concentrations of either architecture produced greater silencing of the targeted gene, but the intact dendrimers consistently proved to be more effective than the fractured architecture. In an investigation into in vivo transfection potentials for various PEIs, Brissaut et al. concluded that the content of primary, secondary, and tertiary amines is only of minor importance, while topology plays a key role in ultimate gene expression (Brissaut et al. 2006). Topology may only become evident above certain molecular weights in polymers. Low molecular weight branched PEIs (<10,000 Da) have been shown to be ineffective for gene delivery (Godbey et al. 1999a). However, cross-linking small PEIs yields carriers with transfection efficiencies similar to larger branched PEIs (Thomas et al. 2005). In consideration of cytotoxicity, such cross-linking yields molecules that are less damaging to cells presumably because degradation (at the ester or amide linkages used for cross-linking) converts the molecules back to small, non-toxic PEI molecules (Thomas et al. 2005). However, this is inconsistent with reports of polycation toxicity being due to extracellular, membrane-damaging effects. The importance of carrier molecular weight inside and outside the cell is certainly worth significant attention when designing novel carriers.

An interesting contrast of linear vs. branched PEI has been carried out both in vitro and in vivo in lung epithelia. In this model in vivo, the 22 kDa linear form has been used to produce higher reporter expression than the 25 kDa branched form of PEI (Wiseman et al. 2003; Brissaut et al. 2006). Here, the presence of mucus and lung surfactants would seem to play a role in which form of PEI was the better gene delivery agent. However, in contrast to lipofection, gene transfer with cationic polymers to airway epithelial cells does not seem to be inhibited by pulmonary surfactants in vitro (Ernst et al. 1999). The differences in PEI architecture are undoubtedly partially responsible for the differences seen in lung transfection efficiencies. Perhaps the architectural difference is responsible for aggregation of PEI—DNA complexes that could affect ultimate transfection efficiency in vivo. The introduction of salt to salt-free PEI—DNA complexes causes aggregation into larger
complexes when linear PEI is used, but complexes remain small when the branched form is used (Wightman et al. 2001). It is evident that considerations that extend beyond transfection of a specific cell must be taken into account when designing non-viral carriers. Different cell types exist in widely different environments, whether they are gastric cells in a low-pH environment, lung or colon cells bathed in mucus, or brain cells in a systematically privileged locale. Specific features of a given delivery vehicle might make that carrier better suited for specific physiological situations.

Architectures

So far in this text, polymers such as PEI, PLL, and various dendrimers have been discussed and grouped under the single heading, “polyacations.” There is a distinct architectural difference between these molecules, though, which stems from the polymerization methods used for each molecule. A hyperbranched polymer such as PEI is produced by an acid-catalyzed chain growth mechanism that has branch sites arising from specific interactions between two growing polymer molecules, with growth termination occurring by “back biting” or intramolecular macrocyclic ring formation (Tomalia and Killat 1985). Dendrimers, on the other hand, are produced in a controlled stepwise fashion to produce predictable and reproducible structures with polydispersity indices near 1.0. In examining parameters that will contribute to a novel, rationally designed gene delivery vehicle, rigorous investigations involving dendrimers are warranted because of the control available in the polymerization process and the reproducibility of molecules between production lots.

There is an array of dendrimeric molecules that can be used for gene delivery. A commonly used dendrimer type includes the PAMAM dendrimers (Figure 4). Various generations have been used for gene delivery, with optimal generations differing by cell type (but generally between G5-G10; Kukowska-Latallo et al. 1996). Spherical G5 PAMAM dendrimers have also been used in vivo with success (Zhong et al. 2008). Other dendrimers, such as PPI dendrimers (Figure 5), have also been used for gene delivery with transfection success showing a dependence upon generation number (Zinselmeyer et al. 2002). Presumably because of the increased density of positive charges at the dendrimer periphery, an increase in DNA binding was observed as dendrimer generation number went up. Cytotoxicity was also seen to be generation dependent.

Other interesting dendrimers that have been used for gene delivery include asymmetric and cyclic core formulations. Asymmetric dendrimers can be produced by conjugating a dendrimeric polycation with a hydrophobic tail (such as α-amino myristic acid (C14) residues) to produce amphipathic, asymmetric dendrimers. One such example is ((ω-εLys)_{15})αAMA)_1 amide (Shah et al. 2000). Cyclic core dendrimers have been produced using 1,4,7,10-tetraazacyclododecane as the core molecule (Cheng et al. 2000). These dendrimers have also been used to deliver reporter genes into various cell types, including COS7 and 293 cells.

Degradation properties of delivery vehicles may be of interest when designing novel vectors. For instance, PEI is not known to degrade, and endocytosis data indicate that linear and branched PEIs remain in the cell at least for the first hour after uptake (similar to PAMAM dendrimers; Seib et al. 2007). In the studies involving hyperbranched poly(amoeno esters), Wu et al. (2005, 2006) found that they degrade more slowly than linear poly(amaeno esters), and that the type of terminal amine had little effect on the rate of hydrolysis and DNA condensation capabilities. Since hydrolysis occurs from within the molecule, it is reasonable to assert that the compact hyperbranched structure prevents accessibility to water molecules, while the linear form is more open to hydrolysis. No report was made as to the degradability of linear vs. branched molecules after complexation with DNA, which could serve to obscure some of the hydrolysis sites in the linear molecule. The hyperbranched poly(amaeno esters) were found to have similar transfection efficiencies to branched 25 kDa PEI in HepG2 cells, but were found to be less cytotoxic than PEI. The team reported little effect of terminal amine type on DNA condensation ability. Keep in mind, however, that while DNA condensation and binding of constructs to cell membranes are both key to successful transfection, they are distinct events.

Partial vector degradation may be of benefit to transfection capabilities in PAMAM dendrimers. Through solvolysis, such dendrimers have been fractured to yield gene delivery vehicles that have greater flexibility, allowing for an increase in particle diameter due to mutual charge repulsion after additional protonation of amines following drops in pH (as might be seen between the early and late endosomal stages of endocytosed particles; Tang et al. 1996). While the total number of protonable amines is reduced by such degradation, the modification yields a polymer that is more dynamic in terms of size and transfection complex architecture. Post-polymerization modifications can add to the robustness of a gene delivery vehicle by imparting very specific properties to address the given hurdles to desired transfection characteristics. Other modifications will be considered next.

Modifications

Combinations and modifications of polymers have been pursued in an attempt to mix qualities of each
constituent, or to temper a characteristic found in a basic formulation. We have already mentioned how low molecular weight PEIs that are not noted for efficient transfection, but are nevertheless relatively safe in terms of cytotoxicity, have been cross-linked in an attempt to produce larger molecules with the gene delivery capability of larger PEIs without the associated cytotoxicity (Thomas et al. 2005). Others have recognized that the dense cationic nature of delivery vehicles is responsible for cell death and have reacted by producing molecules with PEG additions to reduce cytotoxic effects. The hypothesized reduction in cytotoxicity was found to be the case with PEG-block-PEI copolymers (Zhang et al. 2008). However, as with the cationic polymers themselves, the molecular weight of the PEG additions is also of importance. PEG side chains of molecular weight 350 kDa tend to stabilize polymer–DNA complexes, while longer chains reduce transfection efficiency because of greater steric hindrance (Sung et al. 2003). PEG has also been used with linear PEI in a tri-block architecture, producing branched, cationic molecules with strong buffering capacities and relatively good transfection efficiencies for PEI–PEG–PEI 4000–3400–4000 (Zhong et al. 2005). Consistent with unmodified PEI, smaller molecules (PEI–PEG–PEI 2100–3400–2100) were found to have both lower transfection efficiency and lower cytotoxicity. The tri-block approach has also been tried with PAMAM dendrimers, where PEG 3400 was used as the polymeric supporter to produce linked G5 dendrimers (Figure 15; Kim et al. 2004). These constructs were also found to produce transfection efficiencies similar to branched PEI in 293 cells (although their efficiencies were much lower in HepG2 cells), and the PEG again seemed to improve cell viability.

As with PEGylation, modifications in the form of acetylation have been used to reduce cytotoxicity during the transfection process. Also, just as with most modifications, acetylation has been performed on both PEI and dendrimers. When PEI was acetylated at primary and secondary amines to form secondary and tertiary amides, the predictable effects of decreases in zeta potential and buffering capacity per mole of nitrogen and increases in polyplex diameter were observed (Forrest et al. 2004). While the anticipated increase in cell viability was not achieved with the acetylation, increases in transfection efficiency over unmodified PEI were reported. On the dendrimer side, this time with PPI dendrimers, acetyl groups or glycol gallate have been used to modify exterior primary amines, while interior tertiary amines were modified with methyl iodide or methyl chloride to produce quaternized cationic sites in the dendrimer cores (Tack et al. 2006). Fourth-generation dendrimers showed encouraging transfection efficiencies in the presence of serum. Although extensive investigation was performed by the group with the acetylated PPI dendrimers that showed low cytotoxicity, PPI dendrimers (G4) modified with PEG and methyl iodide were chosen and successfully used to deliver ssDNA to nude mice in vitro.

Alkylation has been used with non-traditional gene delivery vehicles to investigate the effect of chain length and degree of alkylation on transfection efficiencies. Poly(4-vinyl pyridine) (PVP) was converted to quaternized, alkylated molecules of C\textsubscript{4}PVP (n = 1–6), as shown in Figure 16 (San Juan et al. 2007). It was found that the length of the alkyl chain was key to transfection potential, with C\textsubscript{4}PVP showing the most favorable gene delivery results. When the length of the added carbon chain was less than four or greater than five, no transgene expression was noted. In addition to chain length, the degree of alkylation of the C\textsubscript{4}PVP was investigated and found to be of importance, with 65% alkylation yielding optimal results. The charge ratios used for transfections were based on the number of quaternized N-alkylpyridinium moieties vs. the number of phosphates in the delivered plasmids, and were held constant at five. This investigation was important, in that it systematically examined the importance of chain length and degree of quaternization/alkylation toward transfection efficiency (although the polymers produced approximately 17% of the transgene expression obtained with a commercial PEI formulation).

Reducing the surface charge concentration for increased cell viability is not the only reason that polymeric carriers might be modified. Several studies have utilized the addition of amino acids to gene carriers with varying results in terms of both cell viability and transfection efficiency. PEI has been modified with histidine, but this modification yielded no remarkable improvements in transfection efficiency vs. unmodified PEI (Mennesson et al. 2005). PAMAM dendrimers (G4) modified with the cationic amino acid arginine showed lower cytotoxicity (in cortical cells) vs. unmodified PAMAM, branched PEI, and Lipofectamine (Kim et al. 2006). These complexes were used to successfully transfact astrocytes, microglia, and oligodendrocytes, cells often considered to be difficult to transfact. Arginine has also been used to modify PPI dendrimers (G2), this time to increase transfection efficiency over unmodified PPI (G2) (Kim et al. 2007). Note that the generation number in this case is lower than that of the PAMAM dendrimers mentioned, which could indicate that the arginine modification produces improved transfection results for only lower generation numbers.

**Modifications with broader effects**

Additional modifications for increased cellular or nuclear entry, without focusing on complex charge,
have been carried out. PEG-star polymers (four branches) with one of five specific cationic peptide sequences have been used with the intent of binding heparin for enhanced cellular entry (Fichter et al. 2008). It was found that tighter heparin binding correlated with higher internalization and transfection efficiency. The transfection efficiency of these modified polymers was less than that obtained with PEI, but the cytotoxicity was also reduced. Another modification to gene delivery complexes has been tried in the form of the inclusion of additional anions during complexation. The efficiency of DNA delivery into cells has been increased by the inclusion of oligonucleotides or dextran sulfate in plasmid DNA solutions prior to complexation with PAMAM dendrimers or phosphorus-containing dendrimers (Maksimenko et al. 2003). This increase in DNA uptake may be due to reduction in the zeta potential of the complexes, which could, in turn, reduce membrane damage. The investigation indirectly supports the importance of charge ratio in successful gene delivery.

For enhanced nuclear entry of gene delivery complexes, Choi et al. (2006) took the approach of modifying PAMAM dendrimers to bind to glucocorticoid receptors. Because glucocorticoids, when bound to their cytoplasmic receptors, are transported to cell nuclei (Adcock and Caramori 2001), the attachment of dexamethasone (a glucocorticoid) to PAMAM dendrimers was employed to assess

![Figure 15. "Dumbbell" polymer with two G5 PAMAM dendrons linked by PEG 3400. This tri-block approach has been used to address cytotoxicity issues.](image)

![Figure 16. Quaternized, alkylated molecules of PVP (C\textsubscript{n}PVP, n = 1–6). Not only is the length of the alkyl chain important to transfection success but also the degree of alkylation also plays a role in the degree of successful gene delivery.](image)
increases in nuclear translocation and transgene expression. Such increases were indeed realized vs. unmodified PAMAM, especially in the presence of serum, and indicate that even after rational design of novel polymers, additional modifications could further improve gene delivery capabilities.

An ambiguous situation exists, however, with regard to the blood proteins such as human serum albumin (HSA). Delivery vehicles that are efficient in binding to and condensing plasmid DNA also tend to bind serum proteins. Investigations involving cationic, anionic, and neutral liposomes have revealed that cationic liposomes bind the greatest amount of serum proteins and show greater accumulation in the liver (Kwoh et al. 1999). This observation applies to polymers, as well. HSA will bind to PLL–DNA complexes in vitro, which, in turn, lowers the zeta potentials of the complexes as expected (Simoës et al. 2000). In fact, HSA has been implicated as the major protein to associate with PLL–DNA complexes in serum, and is suspected of being a major marker for clearance of these complexes from the blood (Simoës et al. 2000). Interestingly, in vitro investigations involving confluent and polarized respiratory epithelial cells has shown that branched PEI, when complexed with DNA in the presence of HSA, more efficiently transfects cells than complexes made without serum, even in the presence of cystic fibrosis sputum (Carrabin et al. 2005). While such counterexamples exist regarding the association of HSA with gene delivery complexes, it is generally accepted that HSA binding is a major barrier to systemic in vivo gene delivery applications. In vitro transfection in the presence of serum should play a role in the testing of novel delivery agents.

Additional effects of gene delivery complexes on host cells and organisms should also be considered. For instance, some gene delivery agents such as branched PEI, chitosan, and modified PAMAM have been noted in cell nuclei during transfection, as noted by confocal microscopy (Godbey et al. 1999b; Huang et al. 2005; Choi et al. 2006). Such nuclear entry could explain observed changes in the expression of endogenous genes post-transfection (Godbey et al. 2001; Omidi et al. 2005). For PPI, the changes in gene expression vary with dendrimer generation (G3 > G2; Omidi et al. 2005). For both PPI and PEI, it was found that cells treated the naked gene delivery agents differently than carrier–DNA complexes (Godbey et al. 2001; Omidi et al. 2005). Another concern is the activation of complement during in vivo transfection (Plank et al. 1996). This could be aggravated by the method of administration, such as via the lungs (Roseneker et al. 2003). In addition, the premature removal of gene delivery complexes from systemic circulation due to complex accumulation in the lungs, liver, kidneys, and spleen should also be considered in evaluating novel vehicles.

Carrier parameters for successful gene delivery

In designing novel carriers for gene delivery, there are a number of parameters that should be considered in order to achieve potentially superior levels of transgene expression. The shape and architecture of the carrier will affect transfection potential, as well as the overall charge density of the vector. Following complexation, the surface charge density (zeta potential) will have direct effects on interactions between the gene delivery complexes and serum proteins/cell membranes; this parameter can be influenced by the ratio of polymer to plasmid, also referred to as the charge ratio. The size of gene delivery complexes will also affect endocytosis and possible nuclear entry. As mentioned in an earlier section, the properties of biodegradability and the cytotoxicity of the vector and its degradation products should be considered. Finally, while affecting size and charge characteristics of the vector and complexes, the conjugation of adjuvants to the delivery vehicle could influence targeting or cell processing after cell entry. The largest trade-off appears to be in the cationic charges contained on a given vector: high charge densities will aid in DNA complexation and membrane association, but they are also associated with cytotoxicity (perhaps via membrane damage) and inactivation by serum proteins. A balance of all of the mentioned parameters must be obtained to produce non-viral vectors that are optimally suited for specific applications, and this balance can be obtained through systematic and carefully designed investigations of the parameters separately and in concert.

Conclusion

The role of macromolecular architecture for passive targeting of drug carriers is a critical factor in designing and optimizing future polymeric carriers. Physical characterization of branched architectures has defined significant advantages over linear analogs including increased solubility, a well-defined size and shape, and a multiplicity of highly accessible end groups. The incorporation of cyclic architectures or hybrid systems offers additional advantages for specific applications, and enables tailoring of the macromolecular properties. For tumor targeting via the EPR effect, research suggests that less conformationally flexible architectures can enable a further enhancement of the tumor targeting over purely linear systems. For gene delivery, the ability to mask toxic amine functionalities and to optimize surface functionality enables the preparation of effective gene delivery agents while minimizing cytotoxicity. However, much research is required in these and other fields involving polymeric carriers to fully understand and take advantage of polymer architecture.
References


