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Dendrimers in Medicine: Therapeutic Concepts and Pharmaceutical Challenges

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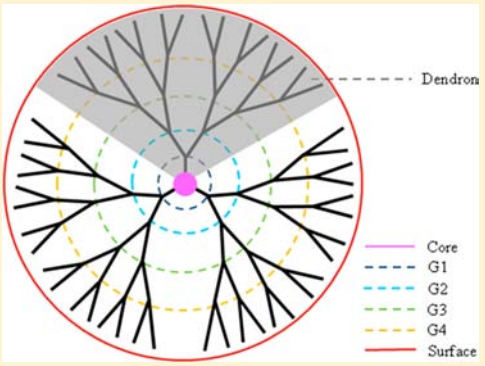
ABSTRACT: Dendrimers are three-dimensional macromolecular structures originating from a central core molecule and surrounded by successive addition of branching layers (generation). These structures exhibit a high degree of molecular uniformity, narrow molecular weight distribution, tunable size and shape characteristics, as well as multivalency. Collectively, these physicochemical characteristics together with advancements in design of biodegradable backbones have conferred many applications to dendrimers in formulation science and nanopharmaceutical developments. These have included the use of dendrimers as pro-drugs and vehicles for solubilization, encapsulation, complexation, delivery, and site-specific targeting of small-molecule drugs, biopharmaceuticals, and contrast agents. We briefly review these advances, paying particular attention to attributes that make dendrimers versatile for drug formulation as well as challenging issues surrounding the future development of dendrimer-based medicines.



1. INTRODUCTION

Dendrimers are well-defined synthetic polymeric architectures with low polydispersity and controlled surface functionalities.[1,2] Dendrimers exhibit three main architectural components (Figure 1). These include an interior core to which dendrons are attached, branching layers (generations) surrounding the internal core, and a multivalent shell. The structure of low generation dendrimers is usually flexible and open, while dendrimers of higher generations are denser and globular.

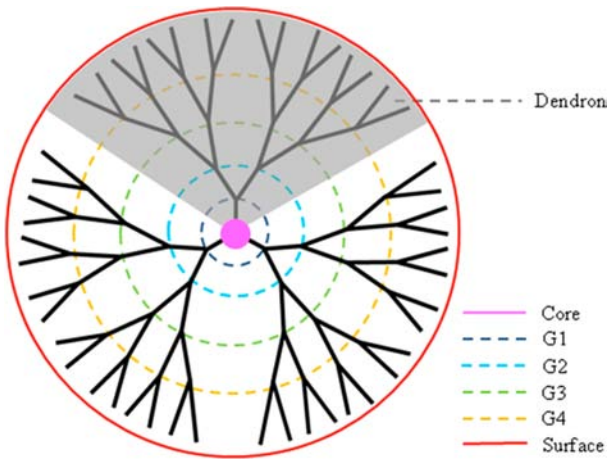


Figure 1. Schematic illustration of dendrimer anatomy. The broken lines indicate different dendrimer generation (G).

Dendrimers, depending on their chemical makeup, have many attributes suitable for drug formulation and administration.[1,3−5] These include narrow molecular weight distribution, nanoscale size and shape-specific characteristics, multivalency, and tunable properties in relation to pH stability, solubility in different media, and biodegradation. For instance, poorly water-soluble drugs may be solubilized and entrapped in the tunable hydrophobic interior cavities of dendrimers (Figure 2), thus offering opportunities for drug formulation, and administration, and improving drug bioavailability, depending on the route of administration.[6−8] The drug-loading capacity of dendrimers as well as drug release from dendrimers may be controlled by adjusting the physicochemical properties of the dendrons as well as dendrimer generation. Furthermore, compared with linear polymers/copolymers, the number of surface groups on dendrimers increases exponentially with increasing dendrimer generation. This attribute not only controls dendrimer size, folding, and shape, but also offers an

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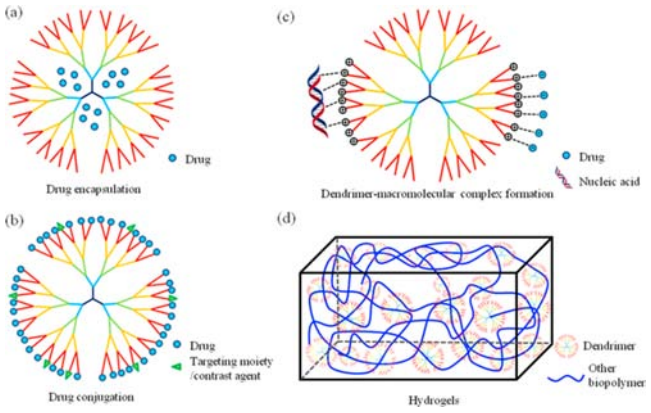

Figure 2. Selected biomedical applications of dendrimers: (a) dendrimers as vehicles for drug encapsulation; (b) dendrimers as multifunctional platforms for conjugation to drug molecules, targeting ligands and reporter molecules (e.g., contrast agents and fluorescent molecules); (c) dendrimers as platforms for nucleic acid compaction and delivery; (d) dendrimers cross-linked to biopolymers for tissues engineering.

unprecedenated ability to modulate dendrimer solubility in both polar and nonpolar media as well as introducing multivalency for conjugation to drug molecules, targeting ligands, molecular sensors, macromolecules such as poly(ethylene glycol)s (PEGs), and other entities (e.g., C12 lauric acid) (Figures 2 and 3).[1,5,9−11] This further paves the way for

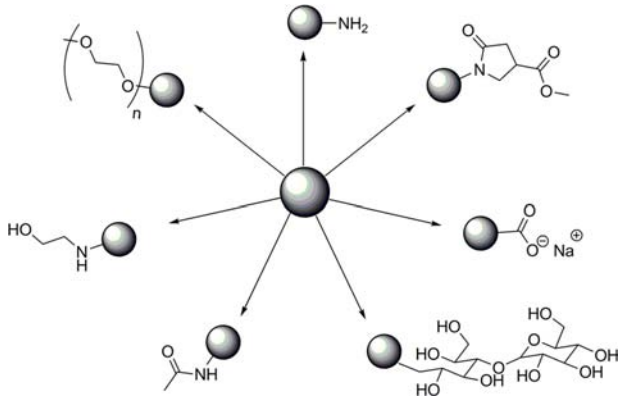

Figure 3. Selection of dendrimer surface functionalities for subsequent surface engineering. The dendrimer is illustrated as the spherical structure.

design of complex multifunctional platforms for theranostic applications as well as personalized therapies. Finally, dendrimers, depending on their surface chemistry and reactivity, can act as "reverse micelles", form complexes with other macromolecules including proteins and biopharmaceuticals (e.g., nucleic acid medicines) (Figure 2), and display inherent antimicrobial activities.

The dendritic properties have introduced a new "nanoperiodic" concept, which proposes a strategy for optimizing drug pharmacokinetics and site-specific targeting with dendrimers through engineering of "critical nanoscale design parameters" comprising size, shape, surface chemistry, flexibility/rigidity, architecture, and elemental composition.[12] The parameter by parameter "structure−activity" mapping outlined in the "nanoperiodic" concept, however, is different from systems approaches in understanding the integrated physicochemical and pathophysiological factors[13] controlling dendrimer performance and responses in the body, and therefore must be considered cautiously. First, dendrimer properties such as surface characteristics, shape, size and aggregation state may change dramatically and heterogenously on guest molecule encapsulation, conjugation, and complexation. Accordingly, in a typical preparation different subpopulations of dendrimer-based medicines may be present and show variable pharmacokinetics, biodistribution, and safety profiles (e.g., immune system recognition and associated responses) following administration into the body with significant deviations from the expected "nanoperiodic" concept. Second, architectural changes (e.g., size and shape) may still occur after dendrimer administration, for example, as a consequence of rapid or slow drug release from dendrimer cavities (or gradual dendrimer component degradation/shedding), resulting in altered drug and macromolecular pharmacokinetics. Third, the overall biological performance of a macromolecular formulation must be analyzed and viewed from the systems biology approach, considering the dynamic interplay of variable cellular and extracellular elements in health and disease progression. Finally, despite the aforementioned attributes that make dendrimers a suitable candidate for prodrug and drug delivery system design, the polymeric nature of dendrimers and their derivatives thereof provide significant challenges from pharmaceutical manufacturing perspectives, which includes material purity, characterization, separation, scale-up, and regulatory issues, and these require addressing. Here, we briefly review recent and promising applications of dendrimer engineering in medicine, including a discussion of dendrimer performance from an integrative biological perspective, and pharmaceutical challenges facing the design and manufacturing of dendrimeric formulations.

2. DENDRIMER TYPES

Dendrimers are typically synthesized by two main approaches. The first is the divergent methodology, where the dendrimer is built from the core molecule and outward. Through this approach the core molecule reacts with monomers bearing one reactive and two dormant groups forming the first generation dendrimer. The periphery of the first generation dendrimer may be activated for reactions with more monomers, and the process can be repeated for several generations. Accordingly, a dendrimer is built layer after layer. However, the chemical reactions in the individual steps have to proceed in very high yields if pure dendrimers of a particular generation are to be obtained. The divergent approach usually forms lower dendrimer generations (G0, G1, and G2) of asymmetric shape and open structures. However, problems may occur from side reactions as well as incomplete reactions of the end groups during synthesis of higher generation dendrimers, thus yielding structural defects and heterogeneous preparations. Nevertheless, as dendrimers grow they adopt a globular architecture. When a critical branched state is reached, dendrimers cannot grow further as a result of the steric constraint imposed by the increasing branch density. This phenomenon is known as the "starburst effect", and is usually observed at high generation. The second synthetic approach is the convergent methodology, where the individual branches of polymeric arms (dendrons) are synthesized first and then attached to a multifunctional core molecule.[14,15] This approach overcomes certain limitations associated with the divergent approach, such as minimizing the occurrence of structural defects and easier purification of the

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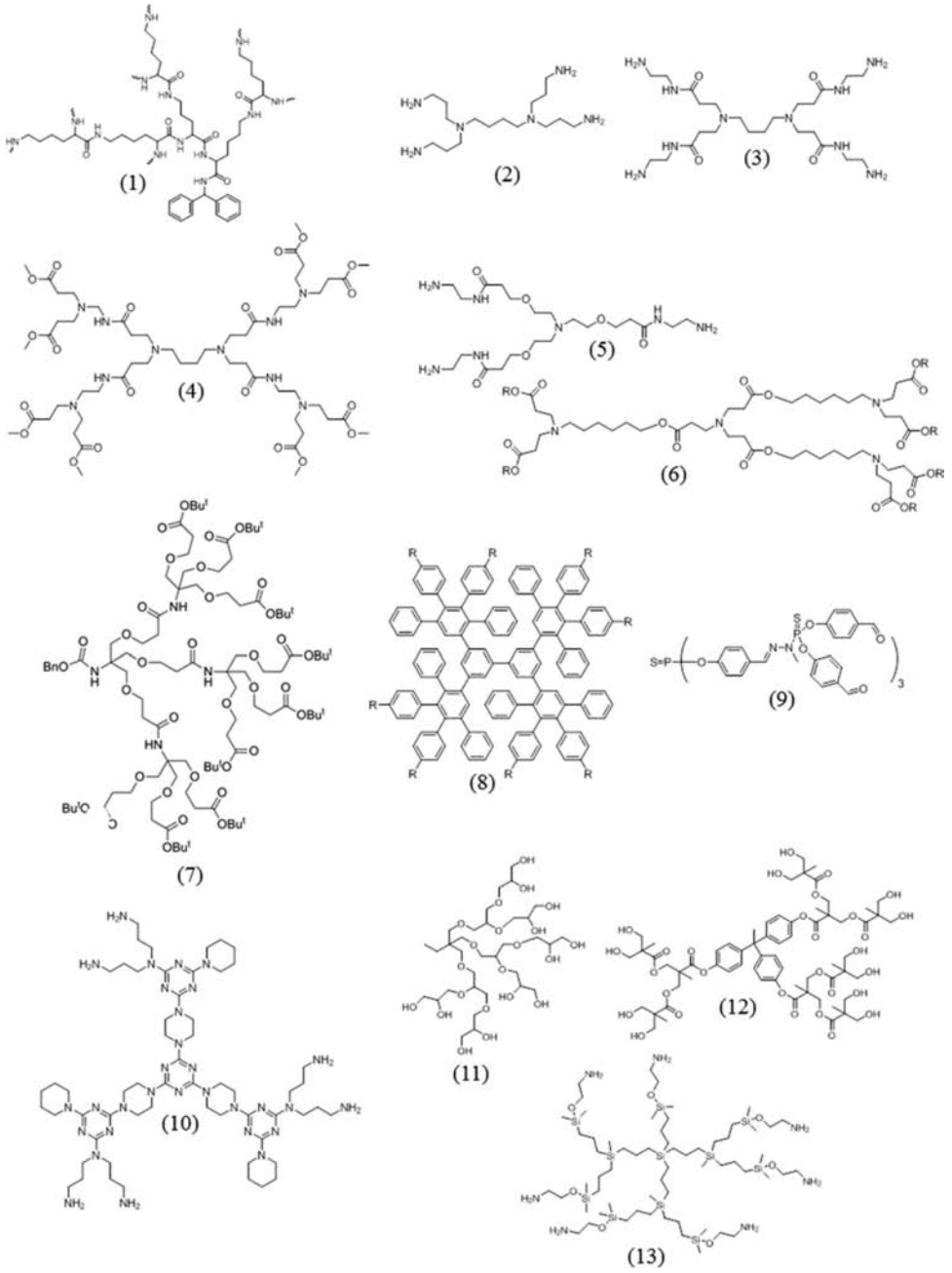

Figure 4. Structure of selected dendrimers: (**1**) partial structure of a lysine-dendrimer with a benzhydrylamine core, (**2**) PPI G0 dendrimer, (**3**) PAMAM G0 dendrimer, (**4**) PAMAM G0.5 dendrimer, (**5**) ether-amine dendrimer, (**6**) ester-amine dendrimer, (**7**) *N*-Tris[(2-{[(tris{[2-(*tert*-butoxycarbonyl)ethoxy]methyl}methyl)amino]carbonyl}ethoxy)methyl]methylamine dendron, (**8**) polyphenylene dendrimer, (**9**) phosphorus-based dendrimer, (**10**) *s*-triazine dendrimer, (**11**) polyglycerol dendrimer, (**12**) polyester dendrimer, and (**13**) silane-siloxane dendrimer.

end product. However, the convergent approach rarely allows for the assembly of high generation dendrimers due to steric issues surrounding the reactions of dendrons with the multifunctional core.

Today, a wide range of different dendrimer families exists with potential applications in biomedical and pharmaceutical arena.[16−19] Some examples are shown in Figure 4. The lysine-dendrimers (**1**) were among the first family of dendrimers synthesized,[20,21] but the first group of dendrimers that became commercially available were poly(propyleneimine) (PPI) (**2**)[22,23] and poly(amido amine) (PAMAM) dendrimers (**3**,**4**).[24] The ether amine (**5**)[25−28] and ester amine dendrimers (**6**)[29−32] are examples of cationic dendrimers with a more flexible core developed for nucleic acid compaction and delivery to mammalian cells. Other interesting species in relation to biomedical applications include architectures derived from *N*-Tris[(2-{[(tris{[2-(*tert*-butoxycarbonyl)ethoxy]methyl}-methyl)amino]carbonyl}ethoxy)methyl]methylamine dendron (**7**),[33] as well as phosphorus (**9**),[34−37] *s*-triazine (**10**),[38−42] polyglycerol (**11**),[43,44] polyester (**12**),[45−48] and silicon-based-dendrimers (**13**).[49−51] The polyphenylene dendrimers (**8**)[52,53] are examples of nonpolar branched macromolecules that have also found applications in many biological settings.

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3. CONJUGATION STRATEGIES IN DENDRIMER ENGINEERING

Bioactive molecules, targeting moieties, and diagnostic probes may be conjugated to dendrimers through different approaches (Figure 5). For instance, the widely used PAMAM, PPI, and

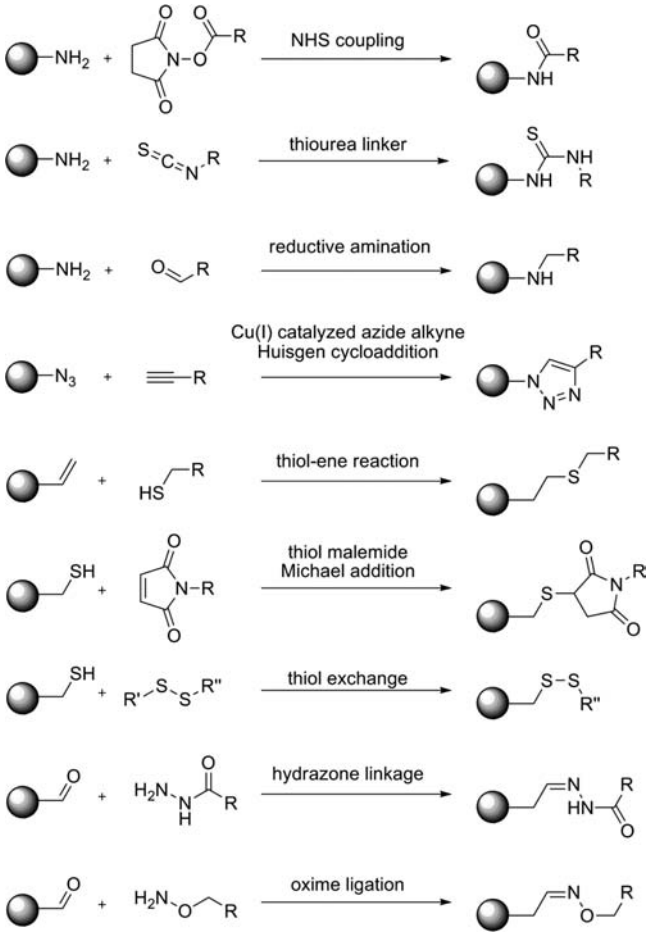

Figure 5. Selection of routine organic reactions for covalent attachment of guest molecules to dendrimers of different surface functionality. The dendrimer is illustrated as the spherical structure. For simplicity, only expression of a single functionality is shown on the dendrimer surface.

lysine dendrimers express primary amino groups on their surface, which may be utilized for simple coupling reactions. The most common approach is the reaction of *N*-hydroxysuccinimide (NHS) activated carboxylic acids with the terminal amines in dendrimers, which proceeds under mild conditions and in good yields. Today, a wide variety of different bioactive molecules with NHS functionality is commercially available, and can easily be conjugated by a one-step reaction to amino-terminated dendrimers.[54−56] Other well-developed approaches include the 1-[3-(dimethylamino)propyl]-3-ethyl-carbodi-imide (EDC) and *N*-hydroxybenzotriazole (HOBt) coupling chemistry,[57,58] and the amine reaction with isothiocyanates, which forms a stable thiourea bond. Reductive amination has also been employed in dendrimer modification, leading to formation of a secondary amine as linker. The conjugation of various carbohydrates to dendrimers via isothiocyanate reaction and reductive amination was extensively reviewed by Liu et al.[59]

In recent years, the concept of "click chemistry", using the Cu(I) catalyzed azide alkyne Huisgen cycloaddition, has further been utilized to engineer new dendrimer structures as well as for conjugation of bioactive molecules to dendrimers.[60,61] Sulfur chemistry has also played significant roles in dendrimer surface modification. Examples include Michael addition of thiols to malemides[55,56] and thiol−ene reactions;[61−64] the latter is referred to "thiol-click" chemistry. Others have introduced degradable linkers such as disulfide bonds,[65] hydrazone,[66,67] and oxime[68,69] for controlled and site-specific microenvironmentally mediated release of conjugated cargo molecules or targeting moiety.

4. DENDRIMERS IN MEDICINE AND PHARMACEUTICAL SCIENCES

4.1. Dendrimers in Drug Delivery. *4.1.1. Drug Encapsulation in Dendrimer Cavities.* The dendrimer interior is particularly suited for accommodating poorly water-soluble drugs, for instance, through hydrophobic interactive forces and hydrogen bonding (e.g., the tertiary amines in PPI and PAMAM dendrimers have a lone pair suitable as hydrogen acceptor).[70] One example is a dendrimer based on glycerol and succinic acid, which was used to encapsulate the poorly water-soluble anticancer drug 10-hydroxycamptothecin (10HCPT) for delivery to various cancer cell lines.[71,72] For instance, this dendrimeric approach not only increased drug uptake by MCF-7 cells by 16-fold compared with the free drug, but further improved cellular retention of 10HCPT based on efflux measurement. Dendrimer surface functionality and properties can further modulate the extent of drug encapsulation in dendrimer cavities. For instance, it has been shown that the extent of adriamycin and methotrexate (MTX) encapsulation in PAMAM dendrimers could increase not only with increasing dendrimer generation, but also with increasing chain length of the surface grafted PEG molecules, where PEG_{2000} was found to be the optimal species.[73] Furthermore, electrostatic interaction between the negatively charged MTX and PAMAM dendrimers may have contributed to higher MTX binding/loading.

Higher generation dendrimers have larger capacities to entrap guest molecules. However, with increasing density of surface projected groups the interior of the dendrimer becomes protected from the bulk solution through "de Gennes dense packing" and conformational folding.[2,71,74−77] The strength of intramolecular interactions between adjacent surface groups as well as the physicochemical conditions of the bulk solution (i.e., pH, polarity, temperature, etc.) play an important role in the "de Gennes dense packing" phenomenon. This feature, coined by Meijer and colleagues as the "dendritic box" approach,[75] has been utilized to modulate dendrimeric guest molecule encapsulation and release rates.

Dendrimers can also accommodate metal salts and nanoparticles. One examples is entrappment of silver acetate in PAMAM dendrimers, which improved the antimicrobial activity of the compound by slowing the release rate of silver ions.[78] Similarly, in another study, silver nanoparticles (AgNPs) were successfully loaded into dendrimers enhancing the anti-inflammatory efficacy of AgNPs and promoting wound healing in conditions with significant inflammation.[79]

While noncovalent drug complexation/entrappment in dendrimers might be the method of choice for solubilization of many bioactive molecules, there might be serious therapeutic issues with this strategy.[80] One serious concern is the inability of the engineered system to control the rate of drug release

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from its core.[81,73,82] For instance, the drug−dendrimer interaction forces may not be sufficiently strong to keep drug molecules in dendrimer cavities once in contact with biological fluids, thus resulting in rapid drug release before dendrimers reach their designated targets.[83] Of particular importance is the partitioning effect in the blood, which must be considered when testing therapeutic efficacy of a dendrimer-based medicine on intravenous adminstration into small animals prior to translational steps into humans. On intravenous injection, dendrimers are diluted more than 3000-fold in the blood pool of an average human compared with a mouse. Accordingly, this may induce rapid drug release from dendrimers (depending on physicochemical attributes of both the drug molecules and the dendrimer void microenvironment) in the systemic circulation of humans, and may account for the lack of therapeutic responses compared with mice models. One the other hand, depending on dendrimer and drug types, drug release from dendrimers may be too slow to exert a pharmacological response. Finally, encapsulation capacity and drug release rates must be adjusted not only in relation to therapeutic dosing and the pathology in question, but also with respect to dendrimer dosing and frequency of administration to avoid dendrimer-mediated adverse effects (e.g., infusion-related reactions, immunogenicity, nephrotoxicity). Some of these limitations could be overcome through covalent conjugation of drug molecules to dendrimers, but this may complicate manufacturing, economics, and regulatory issues. It should be emphasized that hydrophobic drugs may still become trapped in the dendrimer cavities even during conjugation procedures.

4.1.2. Conjugation of Drug Molecules to Dendrimers. The abundance of projected functional groups (Figure 3) on the outer surface of dendrimers provide an unprecedented opportunity for covalent conjugation (Figure 5) of drugs and diagnostic molecules as well as other synthetic and biological entities (Figure 2). Accordingly, this approach offers a means for engineering of macromolecular pro-drug formulations. The type of linker/spacer between the dendrimer and the guest molecules can modulate macromolecular functionality and the release rate of conjugated entities. Common linkers used to covalently attach drugs to dendrimers include acid labile cis-aconityl or acyl hydrazone groups, ester and amide groups, and the cleavable S−S bonds. One example is the cis-aconityl-linked doxorubicin (DOX)−dendrimer conjugate, which displayed successful uptake and DOX release from lysosomes in cancer cells.[84] Depending on the chemical nature of the acid labile groups, the extent of hydrolysis, and hence pro-drug stability in plasma, as well as drug release rates from lysosomal compartments may be controlled further.[85−88] For instance, ester-linked conjugates of Naproxen with PAMAM G0 dendrimers underwent rapid esterase-catalyzed hydrolysis ($t_{1/2}$ = 51 min) in 80% (v/v) human plasma, while the amide-linked conjugated counterpart resisted release. There are other studies that confirm the role of linkage chemistry in dendrimer−drug stability in both plasma and liver homogenate.[85,86] Similarly, ester-linked MTX to PAMAM G5 dendrimer was found to be four times more active than free MTX on cell uptake, while MTX linked via amide bonds to the same dendrimer was less active compared with the free MTX.[87,88] These difference are due to different kinetics of ester and amide hydrolysis by lysosomal enzymes.

There are attempts where drug molecules have been attached to dendimers through disulfide linkages, since disulfide bonds can be reduced by glutathione within the cytoplasm resulting in compartmental (cytoplasmic) drug release. Examples include cationic G4-NH_2 and an anionic G3.5-COOH PAMAM dendrimers with N-acetyl cysteine (NAC) payloads of 16 and 18 molecules per dendrimer, respectively. These conjugates on cellular internalization appeared in the cytoplasm, where the glutathione-sensitive disulfide linker enabled efficient and rapid cytoplasmic release of NAC. The mechanistic aspects of dendrimer appearance in the cytoplasm remain unclear, but may be related to PAMAM dendrimer detergency activity in destabilizing plasma membrane and/or induction of endosome membrane fragment micellization.

Dendrimers have also been used in diagnostic imaging and particularly in the form of architectures comprising covalently linked chelators such as diethylene triamine pentaacetic acid (DTPA) that can hold gadolinium salts.[2,5,89,90] Administration of dendrimer-based contrast agents have many advantages compared with the small molecule formulations, since the latter often show no tissue specificity, suffer from rapid excretion, and require high dosing. In one study, DTPA was conjugated to PAMAM G2 and G6 dendrimers, resulting in the formation of platforms containing 11 and 170 DTPA molecules per dendrimer, respectively.[90] In nuclear magnetic relaxation dispersion studies, the G2 and G6 dendrimer chelates increased the longitudinal relaxivity ($r1$) of Gd(III) ($r1$ is a parameter describing contrast agents efficiency, and a higher $r1$ value correlates with improved MRI signal) to 21 and 34 mM^{-1} s^{-1}, respectively, which in turn was 4- and 6-fold higher than free Gd(III)DTPA (5.4 mM^{-1} s^{-1}). Furthermore, the Gd half-life in the blood corresponded to 40 and 200 min with G2 and G6 analogues, respectively, compared with 24 min for Gd(III)-DTPA.

Finally, dendrimers have also been used for delivery of antigens or T-cell helper epitopes, thus providing a new strategy for vaccination.[91−93]

4.2. Dendrimers as Transfectants. Amino-terminated PAMAM and PPI dendrimers have received considerable attention for nucleic acid compaction and cell transfection.[3,94−96] Due to their polycationic nature these macromolecules form dendriplexes with polyanionic nucleic acids, where their morphology and stability are modulated by the dendrimer genertaion, nitrogen to phosphate ratios, solvent type, salt concentration, buffer strength, pH, and even the sequence of addition.[97,98] For instance, with PPI dendrimers, dendriplex stability and net cationic charge increase with increasing dendrimer generation.[97] Accordingly, dendriplexes from higher generation dendrimers are expected to afford higher transfection efficacy due to their higher cationic charge content, which confers better electrostatic interaction with transmembrane heparan sulfate/chondroitin sulfate proteoglycans. However, transfection studies show otherwise, and gene transfer is more efficient with lower generation dendriplexes.[99] These observations may be due to differences in geometrical and packing constraint in dendriplexes of different dendrimer generation, the extent of dendriplex aggregation in transfection media and/or at the cell surface, the mode of dendriplex internalization and trafficking, and hence nucleic acid release rates, and the contribution of dendriplex type to the dynamic cell death processes.[99−101] Indeed, polycationic PPI and PAMAM are highly cytotoxic, perturbing the integrity of cell plasma membrane, a property similar to both linear and branched polyethylenimines.[102] Some of these detrimental effects may be overcome through surface PEGylation[103,104] or by introduction of other functionalities that improve dendrimer

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inertness in biological milieu. Examples of the latter include surface introduction of 4-carbomethoxypyrrolidone,[105,106] hydroxyl,[107,108] carboxyl,[72] and acetyl[108,109] groups. Other strategies encompass surface engineering with biologically derived moieties such as amino acids[57] or carbohydrates.[110] For instance, when 10% of surface groups of PAMAM G5 dendrimer (128 surface amino groups) were conjugated to PEG3400 chains, the corresponding PEGylated dendrimer/DNA complexes showed lower toxicity and up to 20-fold increase in transfection efficiency compared with native dendriplexes.[111] Similarly, for RNA delivery the mPEG−PAMAM-G4 complexation significantly enhanced functional delivery of RNA into a human melanoma cell line.[112] On the other hand, with hydroxyl modified PAMAM G4 dendrimer the transfection efficiency was poor, presumably compromised by poor interaction with the cell membrane.[113] However, transfection efficacy of such systems may be improved through surface engineering with targeting ligands. For instance, a G4 PAMAM−OH dendrimer was surface functionalized with a synthetic analogue of luteinizing hormone-releasing hormone prior to complexation with a functional siRNA, and the resultant dendriplexes proved functional in both cancer targeting and silencing.[114]

Several recent attempts have combined dendrimers with other macromolecules and molecular assemblies to form better nucleic acid delivery systems and/or improve safety.[115−118] One example is conjugation of α-, β-, and γ-cyclodextrins to the surface of PAMAM dendrimers, which significantly improved nucleic acid delivery and transfection efficiency.[115] The greatest transfection activity was attributed to α-cyclodextrin in a 2.4:1 ratio covalently bound to the dendrimer G3 (approximately 100 times higher for the dendrimer conjugate than for the native dendrimer or a noncovalent mixture of dendrimer and α-cyclodextrin). Similarly, a hybrid was formed between a peptide dendrimer and a lipidic mixture of *N*-[1-(2,3-dioleyloxy) propyl]-*N,N,N*-trimethylammonium chloride and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOTMA/DOPE), which improved transfection up to 50-fold compared with dendrimer−DNA complexes alone, and up to 10-fold over a commercial reagent (Lipofectamine 2000) under optimal conditions.[118] In the absence of lipids, the dendrimer−DNA complexes induced minor cytotoxicity. When supplemented with DOTMA/DOPE, cytotoxicity increased, but remained considerably lower compared with Lipofectamine 2000 and DOTMA/DOPE lipids alone.

4.3. Dendrimers as Antimicrobials. There is an urgent global need for new antimicrobials and alternative mechanisms of action given the rise in resistance among bacteria, parasites, viruses, and fungi. For instance, there are high proportions of antibiotic resistance in bacteria that cause common urinary tract and blood infections in all regions of the world. Indeed, highly methicillin-resistant *Staphylococcus aureus* or multidrug-resistant Gram-negative bacteria cause a high percentage of hospital-acquired infections. To this end, dendrimers with cationic and amphiphilic properties may show antimicrobial activity due to their membrane disrupting properties, which results in microbial membrane solubilization and pore formation, and ultimately death, thus helping to overcome antimicrobial resistance crisis. For intance, PPI dendrimers expressing quaternary ammonium groups on their surface are very potent biocides. The antibacterial properties of these dendrimers depend on dendrimer size, the length of hydrophobic chains in the quaternary ammonium groups, and the type of counter-anion.[119] Since cytotoxicity of cationic dendrimers toward mammalian cells is of concern, the antimicrobial properties of cationic dendrimers may be improved by better surface functionalization. One example is nitric oxide-releasing dendrimers such as *N*-diazeniumdiolate NO-donor-functionalized PPI dendrimers with enhanced biocidal action against Gram-positive and Gram-negative pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus*, and with minimal toxicity against fibroblasts.[120] Another example is poly(lysine) dendrimers modified with sulfonated naphthyl groups (also known as VivaGel).[121] A mucoadhesive gel formulation of this dendrimer is being developed to provide symptomatic relief and to prevent recurrence of bacterial vaginosis as well as preventing transmission of genital herpes (HSV-2), HIV, and other sexually transmitted infections (e.g., human papillomavirus) and is currently in clinical trials.[122,123] There are also examples of amphiphilic anionic dendrimers with improved antibacterial activity and minimal eukaryotic cell cytotoxicity.[124]

4.4. Dendrimers in Tissue Engineering. Dendrimers offer many attributes for hydrogel formation, including multivalency and low solution-viscosity properties, and therefore have found many applications as components of a tissue scaffold for promoting cell proliferation and tissue repair.[125] For instance, dendrimer multivalency allows for high cross-link densities at low polymer concentration, and varied physical properties can be observed based on the macromer structure. Indeed, there are scaffolds based on collagen cross-linked PAMAM G2 dendrimers with improved mechanical properties and higher thermal stability than dendrimer-free scaffolds.[126] Higher proliferation of human conjunctival fibroblasts was further observed in PAMAM dendrimer/collagen-based scaffold than with ethyl-3-(3-(dimethylamino)propyl)carbodiimide cross-linked collagen platforms. There are also examples of dendrimer-based collagen mimetic such as those composed of tris(hydroxymethyl)amino methane (TRIS)$[(\text{Gly-Pro-Nleu})_6\text{-OMe}]_3]_3$.[127] These dendrimers form a triple helix structure similar to collagen with improved thermal stability. Since PAMAM dendrimers can potentially form complexes with Cu^{2+} and Ni^{2+}, collagen mimetic structures based on PAMAM dendrimers may have better advantages in wound healing and bone mineralization.

Low-viscosity aqueous solutions can be injected into cavities of irregular shape to form a well-integrated polymer network. Accordingly, dendrimer adhesives/hydrogels have also found applications in repairing linear and stellate corneal lacerations as well as wounds created during a typical cataract surgery as an alternative clinical procedure to conventional sutures.[128,129]

5. DENDRIMER SAFETY AND TOXICITY CHALLENGES

5.1. Cytotoxicity. There have been numerous studies assessing dendrimer cytotoxicity in different mammalian cell lines. Although not conclusive, these attempts have suggested that the extent of dendrimer cytotoxicity may be dependent on the dendrimer chemical makeup and surface functionality, generation, concentration, exposure time, cell-medium composition (e.g., serum concentration and type), and cell type under investigation. Nevertheless, the mechanistic issues pertaining to dendrimer cytotoxicity are poorly understood. Generally, amino-terminated dendrimers induce more cytotoxicity (based on cell viability measurements) compared with PEGylated dendrimers and species with OH [including TRIS], pyrrolidone, and anionic (e.g., carboxyl) functionality of the same generation and concentration. Presumably, this is a

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function of plasma membrane perturbing and destabilizing properties of the surface projected primary amino clusters in cationic dendrimers, thus initiating intracellular loss of metabolites such as ATP, NADP, NADPH, as well as key metabolic enzymes. For example, in one study both PEG-terminated PPI dendrimers and PPI dendrimers with peripheral neutral acetamide groups induced less cytotoxicity and fewer time-dependent changes in the plasma membrane permeability on human umbilical vein endothelial cells than the native PPI dendrimers.[130] The profound cytotoxicity effect of cationic dendrimers was further shown in a study utilizing a series of melamine-based dendrimers with amino, guanidine, carboxyl, sulfonate, phosphonate, or PEGylated functionalities.[131] On the other hand, PAMAM and diaminobutane dendrimers with surface carboxyl functionality did not induce significant cytotoxicity in B16F10, CCRF, and HepG2 cells up to concentrations of 5 mg/mL.[11] Similarly, poly(ethylene oxide) (PEO)-modified carbosilane dendrimers (CSi-PEO) were not cytotoxic in HepG2 and CCRF cells up to a concentrtaion of 2 mg/mL, but the lowest generation CSi-PEO dendrimer was toxic to B16F10 cells at higher concentrations.[11]

To date, the majority of studies assessing dendrimer cytotoxicity have focused on cell viability determination using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay (and other related tetrazolium compound reduction assays). The MTT reduction is a marker reflecting viable cell metabolism through a mechansim most likely involving electron transfer from NADH or other reducing molecules to MTT.[132] The MTT (and other related) reduction assay readouts are not necessarily reliable when compared with microscopic examination and ATP, DNA, or Trypan blue determinations; it is cell type- and cell passage-dependent and may lead to erroneous conclusions in conditions that affect or modulate cellular metabolism. For instance, when adherent cells in culture approach confluence, metabolism may slow down, and this will affect the rate of MTT reduction into formazan per cell. Furthermore, cell culture conditions such as altered pH, albumin concentration, or depletion of essential nutrients may lead to a change in the ability of cells to reduce MTT. Reducing species such as ascorbic acid and sulfhydryl-containing compounds (e.g., reduced glutathione, coenzyme A, and dithiothreitol) can reduce tetrazolium salts nonenzymatically and interfere with the MTT reduction assay.[132] Nevertheless, the MTT assay is not informative and does not provide a comprehensive view on dendrimer safety and dendrimer-mediated cell-death mechanisms. Indeed, cell death (and repair) processes are multifactorial, dynamic, and integrated, and must be investigated in a coordinated manner by considering the mode of dendrimer uptake and trafficking as well as the effect of dendrimers on biomembrane integrity (e.g., plasma membrane and membranes of internal organelles), metabolic imprints (e.g., mitochondrial oxidative phosphorylation, glycolytic flux), and redox homeostasis in a cell-specific manner.[133−135] Such pan-integrated profiling was recently employed to unravel the underlying mechanisms of PEI size- and architecture-induced multifaceted cell damage and death.[133,134] For instance, in comparison to linear PEI, branched PEIs not only induced greater bioenergetic crisis (through perturbation of plasma membrane as well as mitochondrial networks, impairment of oxidative phosphorylation, and direct inhibition of cytochrome *c* oxidase activity), but further disturbed cellular redox homeostasis through diminished production of NADPH, decreased defense capacity of glutathione, and increased oxidative stress. Metabolic analysis further indicated that on PEI exposure, mitochondria attempt to increase energy production possibly to compensate for energy demanding internalization events and/or energy demanding repair mechanisms arising from PEI-mediated insult on cellular membranes. These processes may be applicable to cationic dendrimers. Therefore, dendrimer cell safety assessment is in urgent need of such approaches, and particularly in relation to partially or fully biodegradable species such as polyether,[11,136,137] polyether-*co*-polyester,[138−141] phosphate,[142] melamine,[38,39,143−146] and peptide dendrimers.[147−149] Indeed, unraveling the mechanism of biodegradable dendrimer-mediated cytotoxic responses is central for careful design and engineering of dendritic macromers for drug delivery and controlled modulation of biological processes.

5.2. Interaction with Blood Proteins and Cells. In contact with blood, dendrimers, depending on their physicochemical characteristics, may interact with a wide range of plasma proteins and blood cells. Studies assessing dendrimer−plasma protein interactions should further consider the stoichiometry of complexation, the molecular hydrodynamic dimensions of the protein in question, as well as the occupied protein volume. Such interactive processes may affect protein structure and functionality, blood cell viability and performance, and dendrimer pharmacokinetics and biodistribution. For instance, recent studies have shown that G5 PAMAM dendrimers, regardless of surface functionality (amino, hydroxyl, and carboxylic functionalities), interact with gamma globulins and alter the secondary structure and conformation of immunoglobulins.[150] Here, neither the type of immunoglobulin interacting with dendrimers of different surface functionality nor the stoichiometry of the complex formation was identified. Whether immunoglobulins could modulate dendrimer clearance via macrophage Fc receptors or through subsequent complement activation and fixation still remains to be elucidated. Others have also studied the interaction between PAMAM dendrimers and other blood proteins such as serum albumin and fibrinogen.[151,152] For instance, PAMAM G4 dendrimer (~4.5 nm in size) and serum albumin (with projected molecular hydrodynamic dimensions of 3.8 × 3.8 × 14 nm) formed complexes with stoichiometry of (4−6):1 and (4−5):1 in the case of human and bovine serum albumin, respectively. Interestingly, such interactions did not affect the overall protein secondary structure suggesting availability of protein domains that can hold up to 6 dendrimer G4 molecules.[151] On the other hand, PAMAM dendrimers (G3, G4, G5, and G5-OH) induced fibrinogen structural and conformational changes, but only the cationic species impaired fibrinogen polymerization ability at high concentrations.[153] In addition, the cationic dendrimers inhibited the activity of clotting factors and fibrinogen in the coagulation of whole blood. In parallel with these studies, others have introduced the notion of "protein corona" on dendrimer contact with plasma and identified a generation-dependent interaction with apolipoproteins, complement proteins, and immunoglobulin chains.[154] Conceptually, this is an ambiguous notion, and perhaps an artifact of incomplete separation procedures, since dendrimers often form complexes with proteins in different stoichiometries depending on protein properties (e.g., hydration and folding states) and hydrodynamic molecular dimensions.

The complement system is a complex network of soluble and membrane-associated proteins and represents one of the major

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effector mechanisms of the innate immune system.[155] The function of the complement in innate host defense is accomplished through highly efficient and tightly orchestrated opsonization, lytic, and inflammatory processes. Synthetic nanoparticles and polymeric entities may trigger complements and a number of consequences ensue from complement activation.[155] These comprise both beneficial and adverse reactions, depending on the extent and severity of complement activation as well as microenvironmental factors.[155−157] Within this context, the interaction between dendrimers and complement system has not received considerable attention. A recent study indicated that PAMAM dendrimers of different functionalities (amino, hydroxyl, and carboxylic) do not activate the complement system in human plasma considerably.[150] However, this observation must be treated cautiously, since the type of anticoagulant (citrate) used in this study for plasma preparation can inhibit activation of calcium-sensitive pathways of the complement system. Accordingly, complement activation by dendrimers through both classical and lectin pathways as well as the amplification loop of the alternative pathway may have been missed. On the other hand, two other studies indicated that amino-terminated PAMAM dendrimers either in free form or complexed with nucleic acids can trigger complements, although mechanistic aspects of the activation processes were not worked out.[158,159]

There are many reports on hemolytic activity of dendrimers, where hemolysis increases in a dendrimer generation- and concentration-dependent manner.[11,147,160−163] Notably, cationic dendrimers are most potent in hemolysis, which may be a combined function of electrostatic interaction and membrane-perturbing properties of these macromolecules, leading to changes in membrane curvature and pore formation. However, in contrast to PAMAM dendrimers, PPI dendrimers with diaminobutane and diaminoethane cores do not show generation-dependent hemolytic activity. Dendrimer-induced hemolysis may be reduced or overcome through surface charge neutralization, shielding, or replacement. For instance, the hemolytic effect of cationic dendrimers is decreased considerably on complexation with DNA, but the dendriplexes can induce heamagglutination after prolonged incubation with red blood cells.[164] On the other hand, PEG-poly(ester) dendritic hybrids do not show hemolysis.[165] Nevertheless, it should be emphasized that the bulk of dendrimer-induced erythrocyte hemolysis assessments were done in the absence of plasma. These conditions underestimate the extent of protection offered by plasma protein, since under physiological conditions erythrocytes are bathed in plasma and dendrimers also interact with certain plasma proteins. Indeed, in one study, the hemolytic ability of cationic dendrimers was significantly reduced in the presence of serum albumin.[166] There are limited studies assessing dendrimer interaction with other blood cell types. For instance, incubation of dendrimers with human blood induces platelet activation, where the extent of activation and platelet responses are dendrimer generation and surface group dependent.[167] Others have shown that PPI dendrimers can decrease the red blood cell count mean corpuscular hemoglobin value, while significantly increasing the white blood cell count.[161,147,162]

5.3. Animal Toxicity and Dendrimer Biodistribution. There are limited studies addressing dendrimer toxicity (on the basis of maximum tolerated dose) and biodistribution, particularly on parenteral dosing.[145,168−171] For example, Roberts and colleagues showed that both unmodified and amino-modified PAMAM G3 and G5 at a dose of up to 10 mg/kg were nontoxic in Swiss-Webster mice.[168] On the other hand, Malik et al. studied biodistribution and toxicological profiles of intravenously injected ^{125}I-labeled cationic and anionic PAMAM dendrimers of different generations in rats. Cationic PAMAM G3 and G4 dendrimers were cleared rapidly from the circulation with predominant distribution to the liver, and presumably sequestered by Kupffer cells.[11] Anionic dendrimers (G2.5, G3.5, and G5.5) showed longer circulation times with generation-dependent clearance rates, where lower generations circulated longer, but again with predominant distribution to the liver. These observations are in line with the kinetics of size- and charge-dependent interception of intravenously injected nanoparticles by the liver macrophages. However, the mode of dendrimer type- and generation-dependent recognition mechanisms by macrophages of the reticuloendothelial system still remains unclear. It is also possible that a proportion of dendrimers are delivered to macrophages via binding to other blood cells such as erythrocytes and platelets. Nevertheless, cationic dendrimers were found to be toxic at high doses.

The capillary endothelium of the glomerular filter and the outer surface of the podocytes, including the filtration slits, exhibit a strong negative electric charge. These sites may play some role in binding to cationic entities of small size and selected shapes. Accordingly, efficient kidney accumulation of PAMAM G3 dendrimers has been reported, which may induce toxicity at higher or repeated dosing.[168]

On intraperitoneal injection, dendrimers can also reach the systemic circulation and their subsequent biodistribution mirrored that seen following intravenous injection.[11] The efficient hepatic interception of dendrimers also suggests a need for surface engineering strategies (e.g., surface PEGylation) if targeting elsewhere is the prime objective. In this respect, development of a PEGylated melamine dendrimer was shown not to induce changes in blood chemistry, animal toxicity, and mortality when injected into mice over short periods of time.[131] However, Neerman et al. conducted in vivo toxicity studies by administrating different doses of melamine dendrimers in mice intraperitoneally, and showed 100% mortality with a dose of 160 mg/kg within 6−12 h of injection. There was no mortality or renal damage at subchronic doses of 2.5 and 10 mg/kg, but the liver enzyme activity became profound at 40 mg/kg.[145]

In spite of the above-mentioned studies, it should be emphasized that on drug encapsulation or complexation, the dendrimer size and shape may change considerably and in a heterogeneous manner, which will ultimately affect formulation (both the dendrimer and the drug) pharmacokinetics and tissue distribution on parenteral injection. Accordingly, pharmacokinetic and toxicity profile of a pharmaceutical formulation that may comprise dendrimer aggregates should not be extrapolated from the studies of parent single-molecule dendrimer preparation. Each pharmaceutical formulation should be studied separately through single and multiple dosing in accordance with the pathology in question. From a therapeutic angle, it is imperative to examine drug bioavailability, volume of distribution, and other pharmacokinetic parameters in relation to disease processes at different dendrimer/drug dosing schedules. Finally, limited studies have shown that PAMAM and PPI dendrimers are weakly immunogenic, and immunogenicity can further be reduced through PEGylation.[161,168,170,172] However, it is possible that on drug conjugation, and dendrimeric size/shape transformation, the

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resultant conjugates may act as haptens and trigger immunogenic reactions.

6. FUTURE PERSPECTIVES

Dendrimers have many physicochemical attributes that make them good candidates for prodrug development or drug delivery applications. In addition, some dendrimers possess inherent therapeutic activities, as in combating certain infectious diseases. However, certain challenges in design and formulation of dendrimer-based medicines remain. The potential ability of dendrimers of different surface functionality to cross the plasma membrane, and perhaps other biological barriers, is of great interest; however, we have yet to unravel the molecular basis of multifactorial mechanisms modulating dendrimer uptake (which may involve adsorptive endocytosis or binding to different classes of scavenger receptors), dynamic intracellular trafficking (including possible transcytosis), and interference with cellular machineries, and their ultimate cellular fates (including degradation processes and efflux rates). This information is important for engineering of well designed and stable dendrimer-based pharmaceutical formulations for drug delivery and controlled drug release at the target site, as well as for dendrimers exhibiting inherent biological activity. Therapeutically, we should further differentiate between formulations comprising predominantly single-molecule dendrimers and those based on dendrimeric aggregates, as their biological performance, including toxicity profiles arising from immunological responses and altered biodistribution, may be different. From a pharmaceutical perspective many questions must be addressed. For instance, what are the true opportunities offered by dendrimers in terms of product performance and life-cycle management? How do dendrimers compare with other systems such as antibody−drug conjugates and micellar systems in terms of formulation attributes including drug payload, homogeneity, shelf life, stability in biological fluids, reproducibility, costs, and manufacturing processes? Indeed, there are still serious challenges with characterization of dendrimers and dendrimer-based medicines from a technical as well as a regulatory perspective. Due to the complex nature of a dendrimer prodrug or a dendrimer-based drug delivery system compared with the parent molecule, a more sophisticated level of testing is necessary to fully characterize the product, quantify each component, and evaluate the relationships and interactions between the vital components. Of immediate importance are validation and development of innovative approaches and quantitative techniques to the analysis of active and inactive ingredients as well as polymeric impurities, and their translation to scale-up manufacturing. The latter also presents unique challenges in pharmaceutical development, particularly with dendrimers that require further surface functionalization with other polymers or biomolecules. Indeed, early identification of important process conditions is critical to achieve key attributes and functions when translated to scale-up manufacturing, since, depending on conditions, the process may lead to altered physicochemical changes in the structure of active molecules and dendrons, and generate substantial amounts of impurities. Considering that dendrimer manufacture often requires multiple process steps, a critical consideration is that the dendrimer-based medicine formulation must be robust to ensure high reproducibility and consistency for scale-up production. Another pharmaceutical consideration is the sterilization of dendrimer-based products, depending on the formulation size distribution, homogeneity, and composition. Typically, sterilization may be achieved through conventional sterile filters, since many dendrimer formulations will have size of less than 200 nm. Yet, another issue is possible endotoxin contaminants and provisions for their removal, depending on the formulation composition, unless aseptic conditions are used throughout, but validation processes are still required. Nevertheless, if the above-mentioned conditions are optimized, and in relation to attributes of a drug-formulated dendrimer, then dendrimers may form a viable base for "in situ" preparation where individual components (dendrimers and drugs) are mixed under appropriate conditions to form complex structures as the final finished product for human use. This approach, by manufacturing individual components, circumvents complex manufacturing steps and developments and may significantly reduce costs.

Finally, limited but promising data are currently arising from early clinical studies with dendrimer-based medicines. This together with further developments in dendrimer scale-up manufacturing processes[173,174] and dendrimer characterization technologies will ensure more interest for human applications through local or systemic administration.

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Notes

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