Exploration of biomedical dendrimer space based on *in-vitro* physicochemical parameters: key factor analysis (Part 1)

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Dendrimers are highly branched, star-shaped macromolecules with nanometer-scale dimensions that can be readily modified with a range of functional groups, thus modifying their physicochemical and biological properties. In nanomedicine, dendrimers can be used as vectors for the targeted delivery strategy of a variety of biologically active agents or can be used as drug *per se*. In the future, it will be necessary to designate and develop 'safe' dendrimers, which is currently a crucial concern. Here, we analyze the key *in vitro* physicochemical parameters to be considered for preclinical evaluation of biomedical dendrimers.

Introduction

Nanotechnology is an umbrella term referring to a multidisciplinary field that covers diverse sciences encompassing biology, chemistry, physics, and engineering based on nanomaterials with sizes ranging from the nanometer to micrometer. It has grown from a pure scientific interest to a major industry, with both commodity and specialty nanomaterial exposure to global populations and ecosystems. Indeed, nanotechnology has received significant attention in recent years in several domains, including the construction industry [1].

To circumvent several drawbacks in the development of drugs, nanotechnology approaches have been developed for the treatment of, for instance, cancer and other chronic human diseases. Generally speaking, the use of nanoparticles (NPs) as nanocarriers improves the bioavailability, targeting, and controlled-release profiles of drugs, both alone and in combination.

Several diverse NP types have been investigated for use as nanocarriers of drugs in several therapeutic fields, but mainly in oncology, in the form of: liposomes, albumin-based particles, biodegradable polymer-drug composites, polyethylene glycol (PEG)-ylated proteins, polymeric micelles, polymer–drug conjugate-based particles, inorganic particles, dendrons, and dendrimers. These latter compounds are a family of nanosized macromolecules, characterized by a highly homostructural branched 3D architecture and compact spherical geometry in solution. Dendrimers represent globular macromolecules, with highly branched 3D architecture, the shape and size of which

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can be controlled precisely [2–5]. Dendrimers represent ideal delivery vehicles, and there are high hopes for their future applications in nanomedicine [6].

Dendrimers have generated a large number of research publications, which has grown exponentially from around 2500 between 2006 and 2013. Approximatively 8300 publications in 2 years, and ~25,000 citations highlighted based on using 'dendrimer' as a keyword. In addition, the number of patents, in different applications, has also grown exponentially from four (between 1984 and 1990) to 739 (between 2005 and 2013) [7].

To date, several preclinical and clinical successes have been reported from dendrimers. For example, Roy *et al*. developed glycopeptide dendrimers as anticancer vaccine candidates [8]. In 2012, Starpharma Holdings started two pivotal Phase III trials for the treatment of bacterial vaginosis with VivaGel® (SPL7013 Gel, astodrimer sodium). This active polyanionic G4-poly(L-lysine)-type dendron has 32 naphthalene disulfonate groups on its surface and has shown potent topical vaginal microbicide activity. Starpharma has now received US Food and Drug Administration (FDA) Phase III approval. Starpharma/AstraZeneca recently advanced from Phase I to Phase II trials with a poly(lysine)dendrimer-based nanocarrier encapsulating docetaxel (DEP® docetaxel) showing superior anticancer activity against several important solid cancer types, including breast, prostate, lung, and ovarian tumors. Importantly, patients treated with DEP® docetaxel showed no neutropenia adverse effects or life-threatening toxicity seen in patients treated with conventional docetaxel formulations [9,10].

Here, we analyze specific structural dendrimer characteristics that are useful in developing nanomedicine.

Specific description of structural dendrimer characteristics in nanomedicine

A schematic of typical dendritic architecture for biomedical applications is illustrated in Fig. 1, using polyamidoamine (PAMAM) dendrimers as example. Four different structural components define dendrimers: (i) a central core; (ii) an interior dendritic structure (named branches) comprising regularly repeating branching units attached to the core, and defining generations G_n, where n is 0, 0.5, 1, 1.5, etc.; (iii) an external surface with the possibility to graft multiple-type functional groups distributed in 3D space; and (iv) void spaces capable of accommodating molecular cargo. The main dendrimers developed in nanomedicine are the PAMAM, PPI, PEHAM, carbosilane, and phosphorus families [11].

Numerous chemical combinations of these four components yield nanomaterials of different sizes, shapes, and internal cores that are ideal candidates for nanosciences, including biological applications. The structural properties of these dendrimers include controllable internal cavities (void spaces) bearing specific species for the physical encapsulation of guest drugs and an external periphery that contains multiple functional groups to facilitate specific targeting and recognition. These fine-tuning chemical modifications allow modulation of the composition, architecture, and cellular behaviors of dendrimers, key properties to improve their *in vitro* and *in vivo* characteristics, such as their biocompatibility from cells to tissues and their pharmacokinetic/pharmacodynamic (PK/PD) activities [12,13].

Taken together, dendrimers used in nanomedicine should be biocompatible, biodegradable, easily targetable, useful for thera-

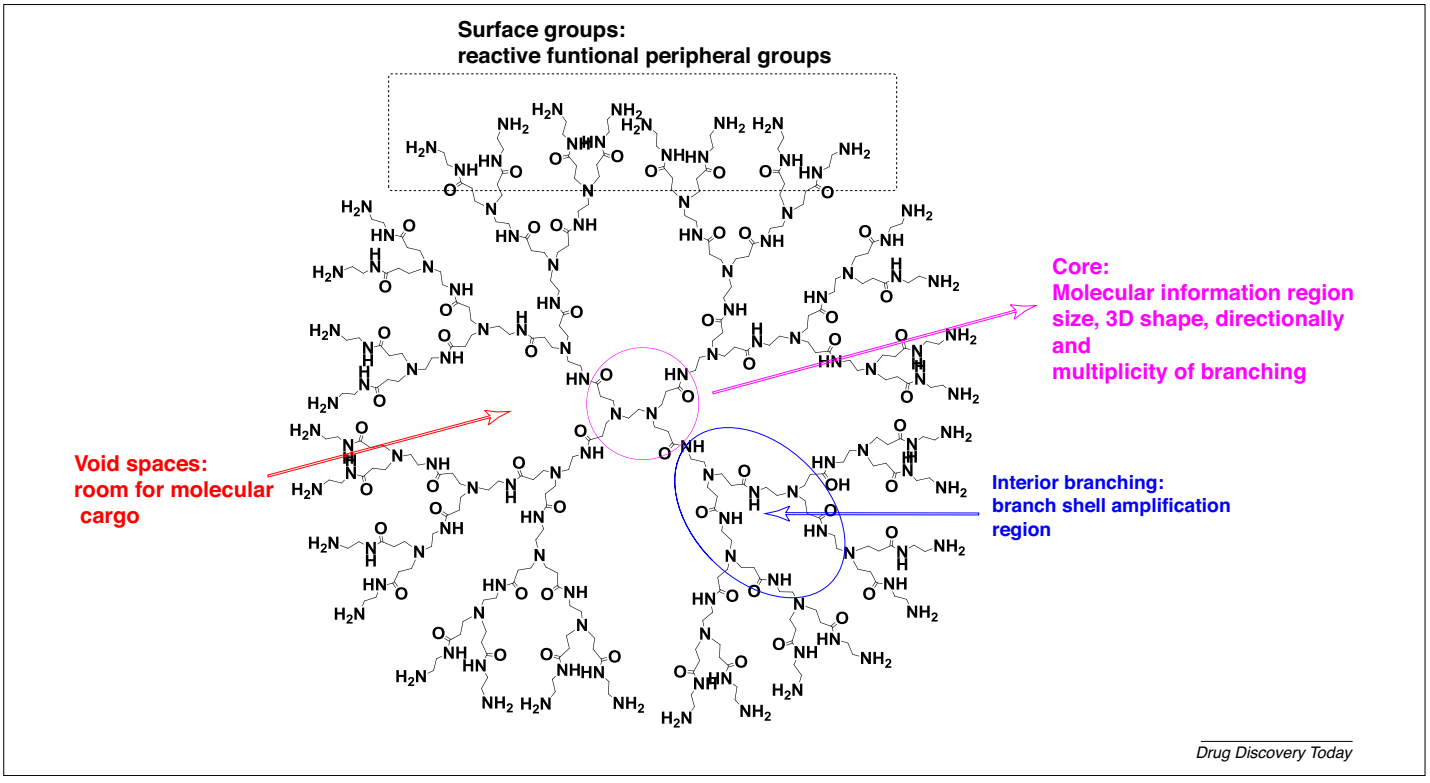

FIGURE 1

Schematic 2D representation of a dendrimer using polyamidoamine (PAMAM) dendrimers as a model with core, void spaces, interior branching, and surface group elements indicated.

peutic and imaging purposes, monodispersed, safe, with high therapeutic windows, non-immunogenic, and with precisely controllable dimensions and architectures. The targeting strategy of dendrimers falls within the thematic of 'precision medicine' that includes the fields of diagnostic (imaging and identification) and therapeutic applications, such as delivery of drugs to exact location, killing cancer cells, bacteria, viruses and so on, and repair of damaged tissues.

However, the main disadvantages of dendrimers are: (i) a lack of knowledge of the effects of dendrimers in biochemical pathways and processes in mammalian systems; (ii) a lack of knowledge of the toxicity and exposure pathways; (iii) cost of development; and (iv) implementation issues. The main advantages of dendrimers in nanomedicine as nanocarriers or as drugs *per se* are: (i) reduce the dose of drug required; (ii) modulation of PK/PD; (iii) increase treatment efficacy related to unsolved medical problems; (iv) control biodistribution of drug; (v) decrease drug adverse effects and reduce mortality and morbidity; (vi) increase drug delivery to the target location; (vii) easy to detect with fluorescent probes, rendering them sensitive diagnostic tools; (viii) improve patient compliance; (ix) reduce unmet medical needs; (x) allow multiple routes of administration [e.g., intravenous (iv), intraperitoneal (ip), ocular, transdermal, oral (po), intranasal, and pulmonary] [14]; and (xi) high drug-loading capacity (local concentration effect).

Recently, researchers suggested the term 'dendrimer space concept' as a new approach for use by medicinal chemists to find original drug-based dendrimers. In this concept, the dendrimer space defines a new 'druggable' cluster that is included in the vast volume of chemical space, taking inspiration from the concepts of 'drug-likeness' and 'druggability', which are fully integrated into the practical drug discovery process [15].

In vitro cytotoxicity and hemolytic activities of dendrimers

The permeability of cell membranes to various families of dendrimers has been studied extensively. In early studies, Tajarobi and colleagues determined the permeability of cationic G0–4 PAMAM dendrimers across Madin–Darby Canine Kidney (MDCK) cell lines and human epithelial colorectal adenocarcinoma (Caco-2) cell monolayers. Caco-2 cells are the most popular cellular model for studies on the passage and transport of molecular entities [16]. This cell line expresses in culture most of the morphological and functional characteristics of small intestinal absorptive cells and is suitable for molecular-permeability screening studies [17]. The permeability of PAMAM dendrimers increased in the following order: G4 > >G0~G1 > G3 > G2 and G1 > >G2 > G0 > G3 > G4 across MDCK and Caco-2 cells, respectively. G0–G2 dendrimers displayed an appreciable permeability that, combined with their low toxic effects on Caco-2 cells, suggests their potential use as water-soluble polymeric drug carriers for oral drug delivery [18]. In a separate study, the overall rank order of PAMAM dendrimer permeability was established as G3.5-CO_2H > G2-NH_2 > G2.5-CO_2H > G1.5-

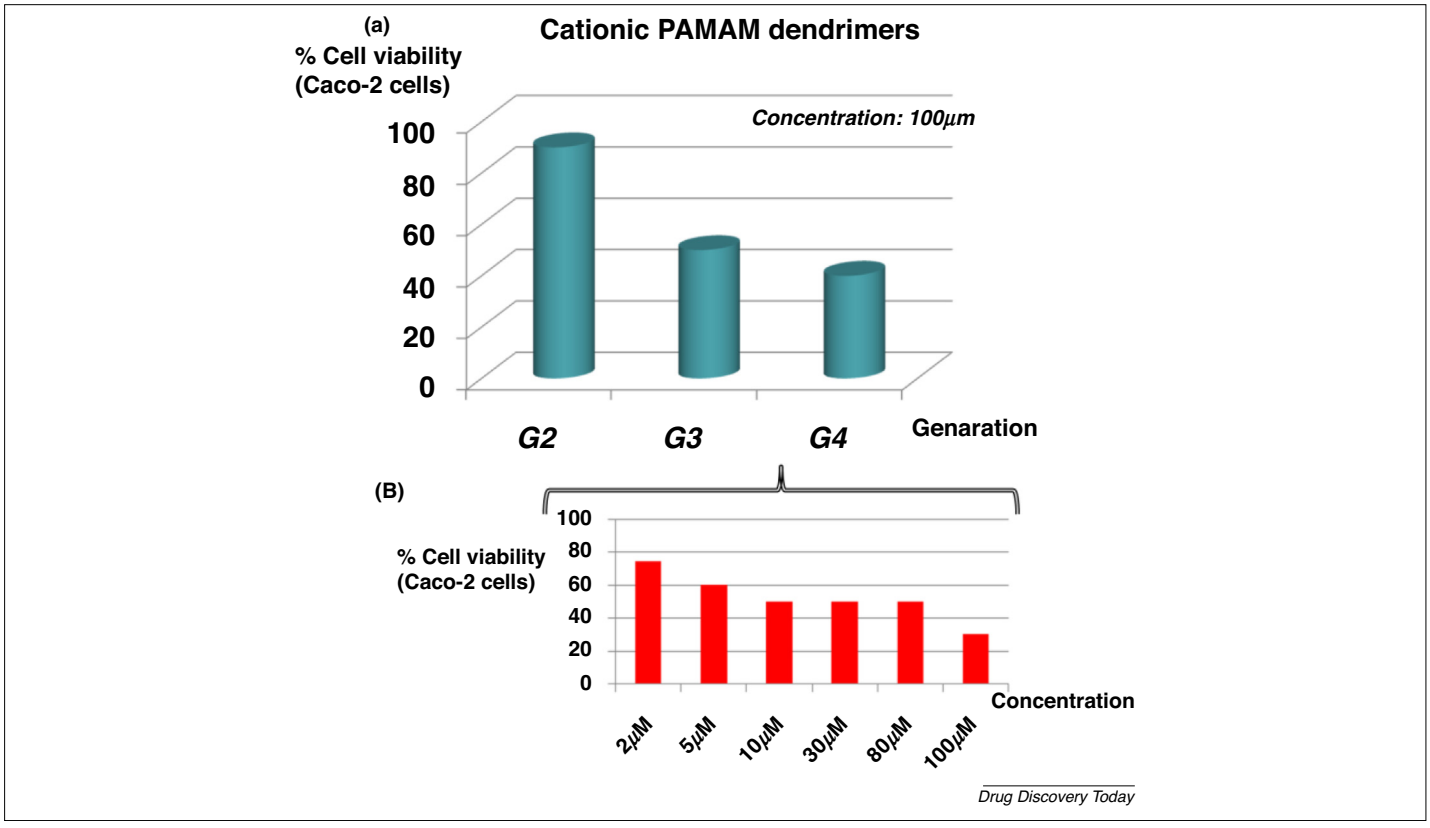

FIGURE 2

Influence of cationic (G2–G4) polyamidoamine (PAMAM) dendrimers on *in vitro* Caco-2 cell viability. The increase in cationic generation decreased cell viability **(a)** and, for a considered generation **(b)**, this viability decreased with the concentration of cationic PAMAM dendrimers.

CO_2H > G2-OH. The strong permeability of cationic PAMAM dendrimers (except G2-NH2) was probably caused by the reversible reduction of trans-epithelial electrical resistance (TEER) and disruption of tight-junction proteins to open this channel [19]. In addition, surface *N*-acylation of PAMAM dendrimers decreased their cytotoxicity while maintaining the membrane permeability [20]. Also, solid-state NMR studies showed that amphipathic dendrimer molecules can be stably incorporated into biomembranes [21]. In addition, improvement in permeability coefficients (Papp), through Caco-2 cell monolayers, of radiolabeled mannitol in the presence of simple dendrimers (G3–5-PAMAM dendrimers), which was more pronounced in the presence of lauryl conjugated dendrimers, was described by Jevprasesphant *et al*. [22].

Numerous studies have been carried out to evaluate the *in vitro* cytotoxicity of dendrimers using different cell lines, concentrations, incubation times, and assay methods. These studies have shown that cytotoxicity depends, to some extent, on the core structure, but is most strongly influenced by the nature of the functional groups on the dendrimer surface [23,24]. The cytotoxicity that induces necrosis of cells (rapid loss of membrane integrity) leading to decreased cell viability and the hemolytic toxicity, which is the breaking off of red blood cells leading to cell lysis and release of hemoglobin into plasma, is dependent on the generation, surface group functionality, and nature of the core and/or branches.

Several studies have highlighted the key role of surface functional groups for the control of cytotoxic properties. Cytotoxicity levels can be decreased by simple modifications of the terminal surface groups, such as PEGylation, acetylation, or the grafting of amino acid, peptide or carbohydrate units. [25]. An alternative strategy used to avoid toxicity is to prepare dendrimer scaffolds based on biocompatible units, such as those produced in known metabolic pathways [26–28]. For instance, high cytotoxicity has been observed for cationic PAMAM dendrimers displaying hydrophilic NH_2 termini. In addition, cationic PAMAM dendrimers induced a significant decrease in cell viability against V79 Chinese hamster lung fibroblasts after incubation for 24 h. Their cytotoxicity level depended on the generation number of the dendrimers: G3, 1 nM; G5, 10 nM; and G7, 100 nM [29]. Further studies from several groups have also highlighted the key role of surface functional groups in the control of cytotoxic properties. Cytotoxicity levels can be decreased by simple modifications of the terminal surface groups. Nitrogen functions with decreased basicity display lower cytotoxicity, because of their decreased interactions with cell membranes. Thus, interaction and membrane bilayer hole creation studies using G7-PAMAM dendrimers against KB cancer cell membranes have been described. Large cationic dendrimers can disrupt cell membranes to facilitate the transport of drugs into cells [30–38]. Recently, Feliu *et al*. published a study of the toxicity of G1-G5 PAMAM dendrimers, with amino (cationic forms) or hydroxyl groups on their surface, for several cell lines, such as

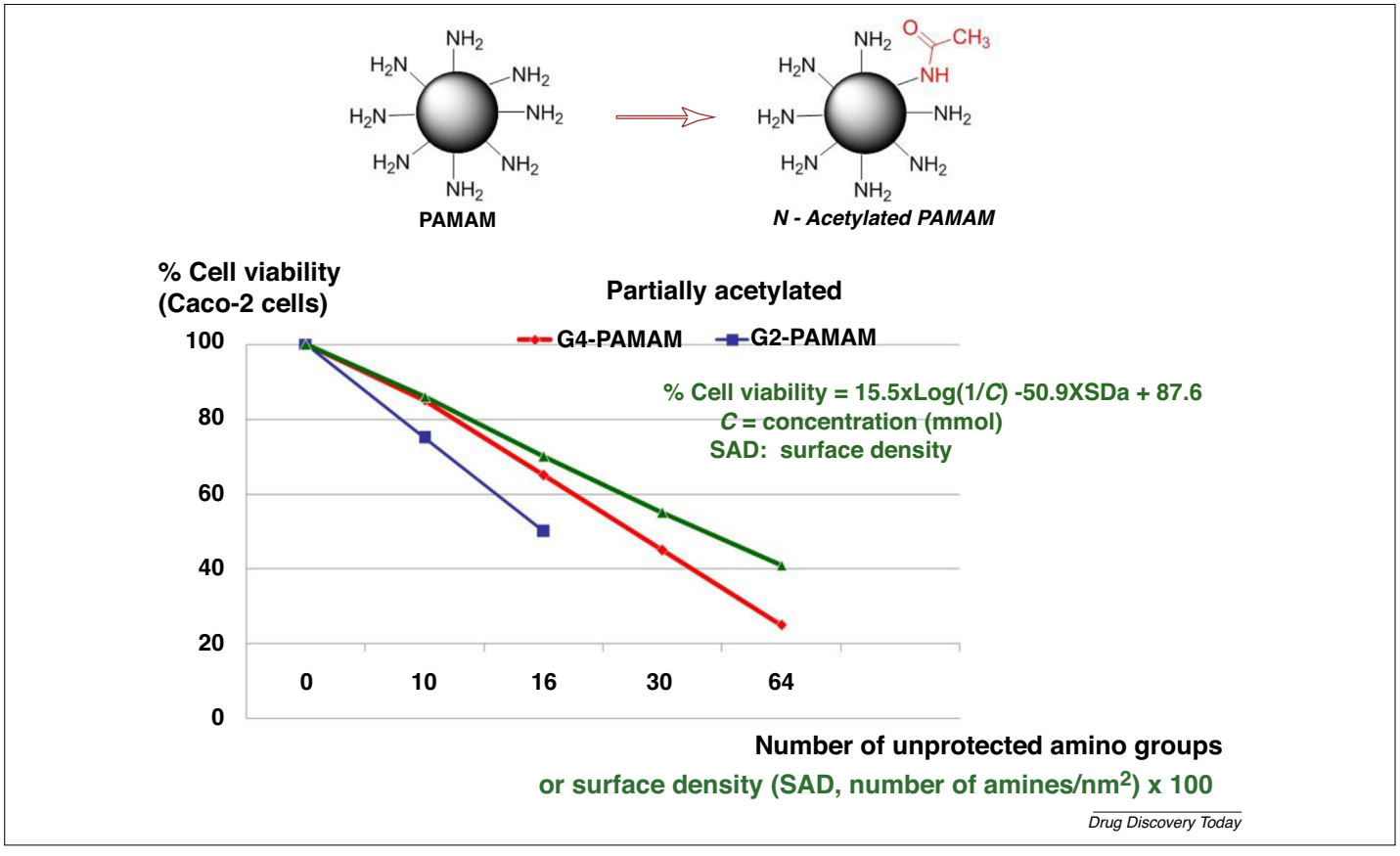

FIGURE 3

In vitro viability of Caco-2 cells with *N*-acetylated G2-4 polyamidoamine (PAMAM) dendrimers. The cell viability decreased when the number of acetylated amino groups on the surface increased.

human cervical cancer (HeLa) and acute monocytic leukaemia cells (THP1), and monocyte-derived macrophages (HMDM) [39]. Excellent biocompatibility was observed for all dendrimers bearing hydroxyl groups, whereas the library of dendrimers with cationic groups exhibited dose-dependent and time-dependent cytotoxicity against HeLa, THP1 and HMDM cells, whereas neutral dendrimers did not.

Interestingly, the concept of dendrimer architectures and *in vitro* membrane bilayer hole formation was analyzed by the comparison of polycationic linear or dendritic polymers includ-

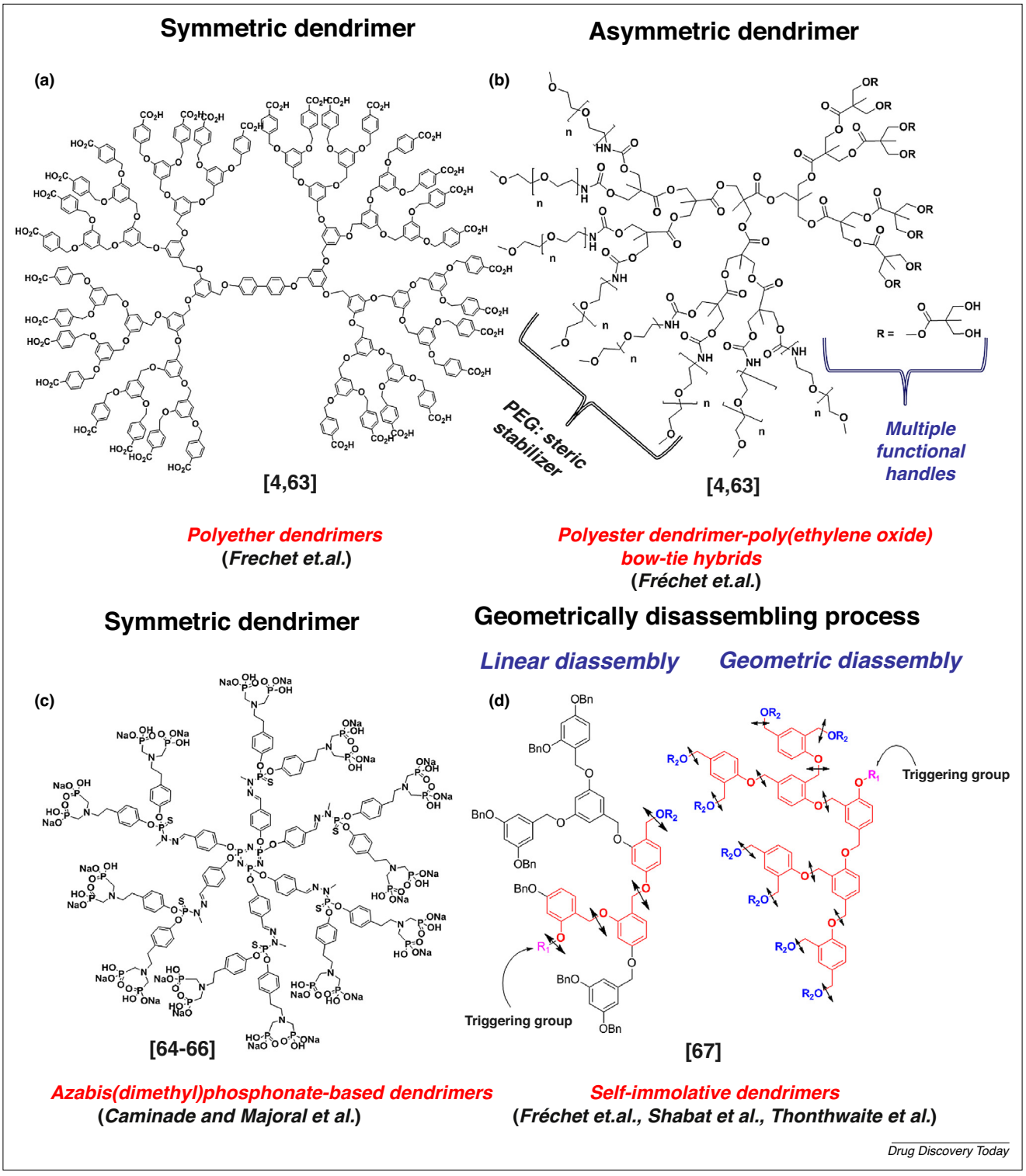

FIGURE 4

Schematic of representative symmetric and asymmetric biodegradable/biocompatible core/branches of dendrimers: (a) polyether dendrimers [4,63], (b) polyester-dendrimers-poly(ethylene oxide) bow-tie hybrids [4,63], (c) azabis(dimethyl)phosphate-based dendrimers [64–66], and (d) self-immolative dendrimers [67].

ing PAMAM dendrimers, poly-L-lysine, polyethyleneimine (PEI), diethylaminoethyl-dextran (DEAE-DEX), and neutral polymers as well as polyvinyl alcohol (PVA), and PEG against KB and Rat2 cell lines [40,41]. Membrane permeability was significantly enhanced by the most cationically charged polymer density, whereas the membrane permeability of PAMAM resulted from its spherical architecture, promoting its interactions with lipid membrane cells and then cell death. PVA and PEG showed no impact on membrane permeability. Similarly, G7-NH2 PAMAM dendrimers, but not G5-NH2 or Ac-G5 dendrimers, were observed to form holes [42].

Figure 2 details the influence of cationic generation (G2–G4) PAMAM dendrimers on the *in vitro* viability of Caco-2 cells. The increase in cationic generation decreased cell viability, and for a considered generation, this viability decreased with the concentration of cationic PAMAM dendrimers. The same authors also reported the role of PAMAM dendrimer concentration and generation (G2, G4, and G6) on cell growth and cytotoxicity in HEK293T and HeLa cell lines [43].

Figure 3 details the *in vitro* viability of Caco-2 cells in response to *N*-acetylated G2–4 PAMAM dendrimers [20]. Cell viability decreased when the number of acetylated amino groups on the surface increased.

Hematological toxicity of dendrimers appears to be a major concern for dendrimers administered intravenously. Hemolytic activity, which is the rupture of red blood cells, can be studied *in vitro* by the evaluation of hemoglobin release across cell membranes, indicating potential cell membrane damage. Free cationic terminal groups of dendrimers interact with red blood cells leading to hemolysis. Hemolytic toxicity of dendrimers such as PPI, PAMAM and poly-L-lysine dendrimers has been investigated [28]. For instance, cationic PAMAM dendrimers showed generation-dependent hemolytic activity: G4 > >G3 > >G1 > G4. PAMAM dendrimers for the delivery of 5-FU showed hemolytic toxicity (~15–17%) [44]. G3 PAMAM and G3 PPI dendrimers displayed hemolytic effects above a concentration of 1 mg/ml [45], whereas, in a separate study, G4 and G5 PPI dendrimers showed hemolytic toxicity of 35.7% and 49.2%, respectively [46].

G4-poly-L-lysine dendrimers induced hemolytic toxicity (~14%) [47]. However, no generation-dependent activity was observed for cationic PEI-based diaminobutane (DAB) dendrimers. In general, higher-generation cationic dendrimers induced greater hemolytic toxicity, which might be attributed to their greater overall cationic charge. By contrast, anionic PAMAM dendrimers and PEGylated melamine dendrimers did not display any hemolytic activity. In addition, no morphological cell changes were observed for half-anionic G3.5–9.5 PAMAM dendrimers (CO_2H-surface groups) [48] (reviewed in Ref. [49]).

The most common strategies for the development of dendrimers with lower cytotoxicity are the following: PEGylation; use of

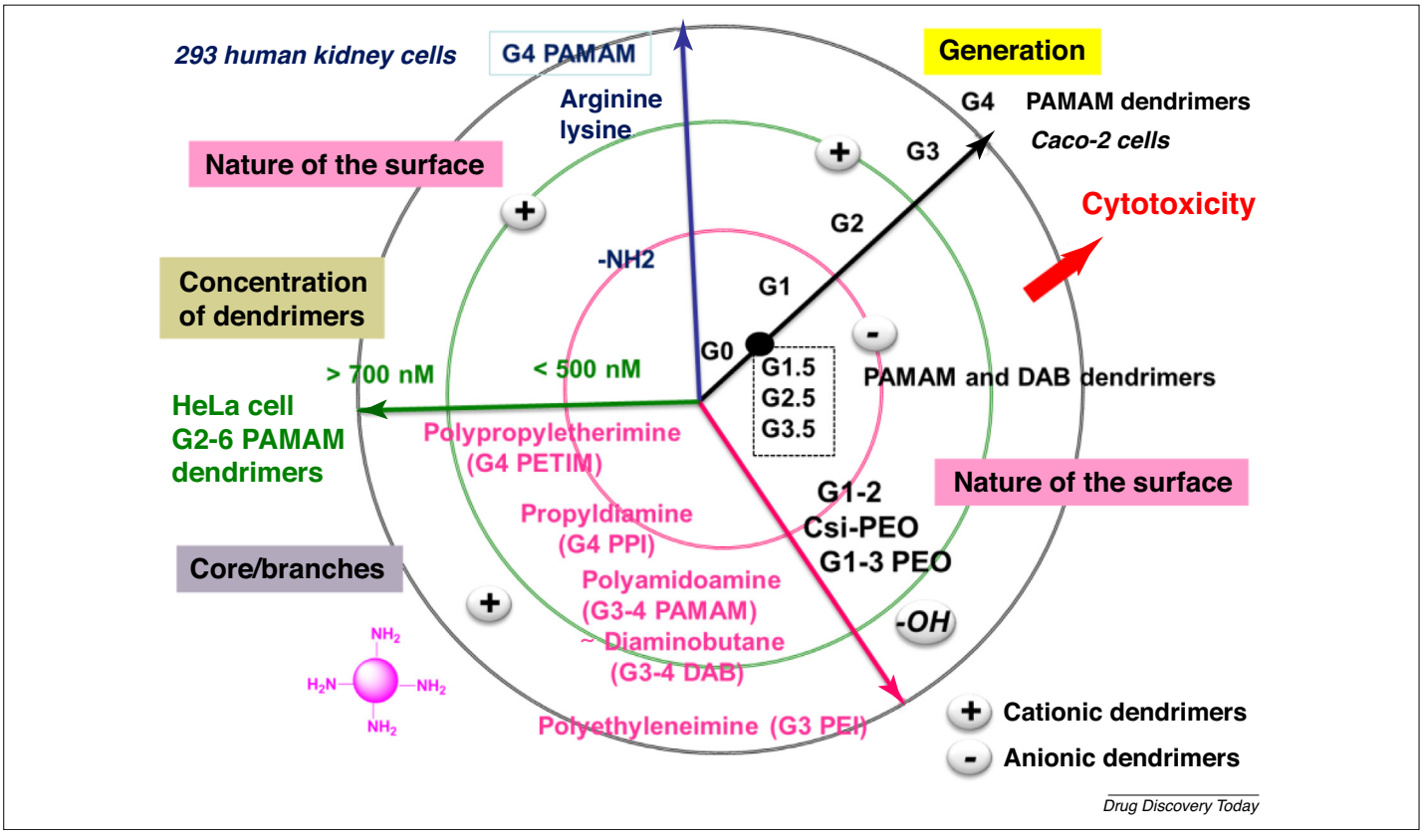

FIGURE 5

Schematic representation of the *in vitro* cytotoxic effects of dendrimers based on generation, surface characteristics, core, branches, and concentration of dendrimers. The *in vitro* cytotoxicity of polycationic dendrimers increases with their generation, whereas polyanionic or neutral dendrimers show no/low cytotoxicity regardless of the generation. The influence of the core/branches on the *in vitro* cytotoxicity against cells follows the order: PETIM < PPI < polyamidoamine (PAMAM) and polyethyleneimine (PEI)-based diaminobutane (DAB) < PEI.

carbohydrate engineered dendrimers; acylation; use of anionic dendrimers; and grafting of amino acids, such as phenylalanine and glycine or peptides [25,50,51].

An alternative strategy to avoid toxicity is to prepare dendrimer scaffolds based on biocompatible units, such as those produced in known metabolic pathways. To date, the main biocompatible dendrimeric scaffolds are: polyether dendrimers [52,53]; bow-tie dendrimers based on PEG and bis-hydroxymethyl-propionic acid [26]; polyester dendrimers [27]; PEI dendrimers [54], polyether-copolymer dendrimers [55], phosphate dendrimers [56], melamine dendrimers [57], 3,4-dihydroxy-L-phenylalanine (L-DOPA) dendrimers [58], peptide dendrimers [59], phosphorus-based dendrimers [60], sugar-based scaffolds [61], and polyurea dendrimers [62]. A nontoxic polypropylimine dendrimer, called PETIM, has been also described [28].

Figure 4 presents representative symmetric and asymmetric biodegradable/biocompatible core/branches of dendrimers. For example, polyether and polyester dendrimer-poly(ethylene oxide) bow-tie hybrids were developed by Fréchet *et al.* [4,63], whereas phosphorus dendrimers were developed by Majoral and Caminade [64–66]. In recent years, several groups simultaneously developed an original strategy, the self-immolative dendrimer strategy, involving the dissociation of dendrimers for molecular amplification as dendritic prodrug systems [67]. The starting molecule was equipped with a focal trigger, which, once activated by a single activation, initiated the fragmentation process (i.e., complete breakdown of the linker) inducing the spontaneous release of all the end-group molecules, which can be biologically active compounds, in a domino-like manner. Figure 5 provides a schematic representation of the cytotoxicity effects related to the generation [36], surface characteristics [25,37,68], nature of core and branches [38], and concentration of dendrimers used. The influence of the generation and the nature of the surface of dendrimers can be demonstrated as follows: the *in vitro* cytotoxicity against Caco-2 cells of polycationic dendrimers increased with dendrimer generation (e.g., PAMAM), whereas polyanionic dendrimers showed no or low cytotoxicities regardless of the generation. Cationic dendrimers (e.g., PAMAM) grafted with arginine, lysine, or amidine groups showed higher *in vitro* cytotoxicity against, for example, 293 human kidney cells. Anionic dendrimers [e.g., PAMAM, diaminobutane dendrimers (DAB)] showed low cytotoxicity against cell. Dendrimers such as PAMAM, PPI, or carbosilane dendrimers with poly(ethylene oxide) (PEO) groups grafted onto their surfaces, resulting in, for example, Csi-PEO dendrimers, or with the introduction of hydroxyl terminated PEO groups on their surface, displayed low *in vitro* cytotoxicity against cells. The influence of the core/branches on the *in vitro* cytotoxicity against cells follows the order: PETIM < PPI < PAMAM and DAB < PEI.

Concluding remarks and perspectives

Taken together, physicochemical properties, such as charge, surface properties, shape, and size, of NPs in general and dendrimers in particular, as well as the route of administration, strongly influence PK by control ling absorption, distribution, and elimination processes. The final goal is the improvement of the plasma residence time and half-life of dendrimers, whether as nanocarriers or not (e.g., as drugs), as well as their tissue permeation, delivering the drug to the target tissue and avoiding adverse effects. Most dendrimers are used as nanocarriers of small and large molecules, genes and peptides. to: (i) enhance the therapeutic potency of drugs by improving their stability under physiological conditions, improving aqueous solubility, improving PK/PD behaviors, preventing recognition by macrophages, and elimination by the reticuloendothelial system, increasing their circulation half-life and selective passive diffusion via the enhanced permeability and retention effect [69], and overcoming low oral bioavailability; (ii) carry out targeted drug delivery; and (iii) allow nonclassical routes of administration.

It is clear that 'safe', nontoxic dendrimers will be mandatory for their clinical developments as both drug-delivery systems and nanodrugs. The two main strategies to avoid dendrimer toxicity are based on the design and synthesis of a biodegradable/biocompatible core and branching units, masking peripheral charges by using surface engineering approaches.

For go/no-go decision-making by project teams earlier during the development process, a list of the desired basic requirements for a dendrimer to become a clinical candidate and to move towards investigational new drug (IND) submission has recently been published [70]. To this end, here, we have outlined and analyzed fully the key parameters to be considered to secure the development of dendrimers for testing in clinical trials and to enhance their translation into the clinic.

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