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Review article

Non-covalent formulation of active principles with dendrimers: Current state-of-the-art and prospects for further development

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ABSTRACT

During the last three decades, dendrimers, nano-sized highly-branched fractal-like symmetrical macromolecules, have been intensively studied as promising candidates for application as drug-delivery carriers. Among other important characteristics arising from their unique and highly-controlled architecture, size and surface properties, the possibility of hosting guest molecules in internal voids represents a key advantage underlying the potential of dendrimers as non-covalent drug-encapsulating agents. The impressive amount of accumulating experimental results to date allows researchers to identify the most important and promising theoretical and practical aspects of the use of dendrimers for this purpose. This review covers the main factors, phenomena, and mechanisms involved in this drug-vectorization approach, including mechanisms of non-covalent dendrimer-drug association, dendrimer-dendrimer interactions, as well as biological properties relevant to the host dendrimers. A discussion is then provided to illustrate some successful existing formulation strategies as well as to propose some new possible ones to optimize further development of the field.

1. Introduction

By and large, the use of inexpensive excipients capable of solving bioavailability problems for FDA-approved active principles (APs) is considered to be the most economical approach over the development of new more-efficient drug entities [1–3]. Therein, particular attention has been paid to excipients acting as encapsulating agents at the nano and sub-micron scales, able to improve the delivery of APs to specific pathological sites.

In this context, dendrimers, highly branched fractal-like symmetrical macromolecules (Fig. 1), described first in 1978 by the group of F. Vögtle [4] deserve more attention. Currently, thousands of different dendrimer structures are known, grouped into > 50 families [5] and are sometimes also less commonly identified as *arborols*, *cascade molecules*, *cauliflowers*, *starburst polymers*, *star shaped nanomolecules*, and *molecular asterisks*[6]. Advantages include, firstly, that dendrimers are characterized by well-defined architectures that potentially insure a very high level of reproducibility in both preparing AP-formulations, and in their pharmaceutical effects after administration. Secondly, being monomolecular structures, dendrimers should not present the problems of low colloidal stability, especially, in biological media commonly observed with nano-formulations. Thirdly, the presence of multiple functional groups at the periphery allows the fine-tuning of surface properties, *e.g.*, chemically grafting substituents capable of

Abbreviations: AFM, atomic force microscopy; AP, active principle; BBB, blood-brain barrier; BCSF, blood-cerebrospinal-fluid barrier; BHBA, 2,2-bis(hydroxyl methyl) butyric acid; BTCA, butane-1,2,3,4-tetracarboxylic acid; Caco2, heterogeneous human epithelial colorectal adenocarcinoma cells; CF, cystic fibrosis; DHBA, 3,5-dihydroxybenzoic acid; DNA, deoxyribonucleic acid; DLS, dynamic light scattering; DOX, doxorubicin; DPMA, poly(*N,N*-dimethylaminoethyl methacrylate; EPR, enhanced permeability and retention (effect); FA, folic acid; FDA, Food and Drugs Administration Agency of the US government; FTIR, Fourier transform infrared spectroscopy; 5-FU, 5-fluorouracil; G, generation number of dendrimer; GSH, glutathione; HAIYPRH, transferrin T7 receptor specific ligand; HCEC, human corneal epithelial cells; HUVEC, human umbilical vein endothelial; ITC, isothermal titration calorimetry; ITZ, itraconazole; KB, human epidermoid carcinoma cells; LFC131, peptide Tyr-Arg-Arg-Nal-Gly; MTX, methotrexate; NCE, new chemical entity; NMR, nuclear magnetic resonance spectroscopy; PAMAM, poly(amidoamine) dendrimers; PFD®, mark of dendrimers produced by Polymer factory (Stockholm, Sweden); PEG, poly(ethylene oxide); PEI, polyethyleneimine dendrimer; PEPE, polyester-*co*-polyether dendrimer; PPI, poly(propyleneimine) dendrimer; pORF-hTRAIL, human tumor necrosis factor produced by InvivoGen (San Diego, CA, USA); PTX, paclitaxel; RBC, red blood cells (erythrocytes); RGD, arginine-glycine-aspartic acid peptide; ROS, reactive oxygen species; SEM, scanning electron microscopy; siRNA, small interfering ribonucleic acid; *t*-BOC, *tert*-butoxycarbonyl; TRIS, tris(hydroxymethyl)-aminomethane; UV, ultraviolet radiation; YLFFVFER, (~H6) breast cancer targeting peptide

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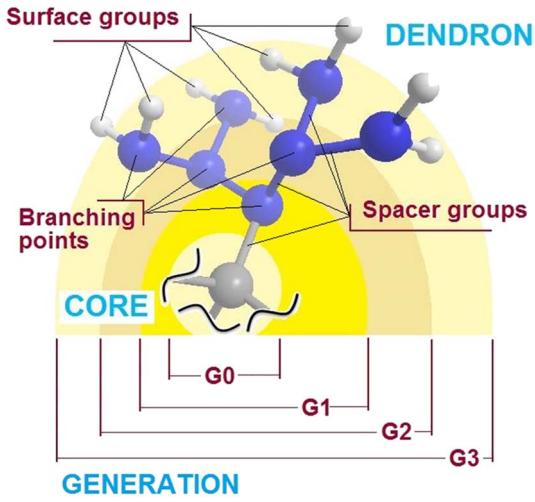

Fig. 1. Schematic representation of the general molecular architecture of dendrimers. A typical molecule of dendrimer consists of a plurifunctional central core and dendrons that determine its overall globular hyper-branched structure. The dendrons attached to the core are composed of spacer groups and branching points covalently linked in a regular arborescent geometry, increasing geometrically the number of the structural elements within each generation, G (each structural layer between the core of G0 and the next branching point).

improving the solubility in selected media, or using molecular markers to facilitate detection and bio-distribution mapping, or inclusion of biologically active entities such as APs and/or ligands to target specific receptors. Fourthly, depending on their generation (G) and chemical structure, dendrimers can carry internal cavities capable of hosting guest molecules of appropriate size and nature, thereby functioning as a 'unimolecular box' [7–14].

The methods of association of dendrimers with APs fall into two main approaches: (i) the covalent grafting of APs to the dendrimer's periphery, and (ii) non-covalent AP-dendrimer interactions. The first approach of covalent grafting is characterized by a higher control over AP-loading as well as by a higher stability of resulting AP-dendrimer hybrid macromolecules. The absence of a spontaneous AP-release in systemic blood circulation is often viewed as the pivotal factor in favoring this approach over the non-covalent association [15,16]. In addition, AP-covalent grafting allows the transport of large APs, such as siRNA [17], ribosome-inactivating proteins [18], as well as the combinatory insertion of APs and ligands specific to cell-receptors [19–23]. An adequate selection of linker groups between APs and dendrimers, providing sensitivity to pathological microenvironments, can potentially lead to a well localized AP-release [24–28].

Despite these seemingly obvious advantages, effective AP-covalent grafting is still, however, very challenging. Above all, there are difficulties in identifying a physical parameter (pH, temperature, chemically active substance, *etc.*) specific to the pathological site only, which, at the same time, will be sufficiently active to break the strong bond between the AP and the dendrimer surface. This often leads to a non-targeted or too-slow drug release severely affecting *in vivo* efficacy [15,29–32]. The use of linkers can also cause the release of less active AP-derivatives [24,33]. Being covalently attached to the dendrimer surface, APs are also more exposed to external factors, increasing the possible risks of transformations before reaching their site of action [33]. An economic aspect is also not to be overlooked since covalent conjugation requires additional chemical transformation and purification steps, which inevitably increase the cost of the final product [15]. Finally, once chemically modified, the AP becomes a *New Chemical Entity* (NCE) that requires an additional approval process [34], so perhaps one of the most promising opportunities for non-covalent systems is that the AP, while bound for transport and release, is still chemically identical to the free AP, and free from any side reactions of breaking a covalent bond to release. These non-covalent 'soft-bonds', such as ionic attachments, hydrogen bonds, or halogen bonds, are also bound by far lower energy interactions ideally sufficient for loading, transport, and stable to premature release, but then requiring only much more gentle conditions to release, more mild or subtle shifts in local equilibria, or *via* triggers such as visible light. In many aspects, the philosophical context of such a 'soft-bonded' approach mimics many natural transport systems preferred by biology, so can be thought of as a bio-inspired design approach, and a 'more natural' motif.

Comparatively speaking, the approach of non-covalent association of APs with dendrimers excludes any NCE formation [15], and is generally characterized by the simplicity of the formulation processing and can lead to an increased solubility of hydrophobic APs through the formation of affinity complexes [35–49], to the reduction in systemic cytotoxicity [46], as well as to an improvement in the bio-distribution *in vivo* after parenteral administration [43]. In some cases, the increase in bioavailability of oral drug formulations was also reported [29,38]. The major drawbacks of this non-covalent formulation approach are mainly limited to a relatively low stability of AP-dendrimer complexes in the systemic circulation, which often results in too-rapid and uncontrolled release (often called 'burst release') of the AP-payload, and consequently, in an improper drug-targeting ratio [15,16]. Despite this limitation, recent progresses in dendrimer chemistry and pharmacology [6], as well as some considerations that will be developed below, give hope that in the near future this non-covalent approach will represent one of the most viable future AP-vectorisation strategies.

The primary goal of this review is to provide a general overview of the use of dendrimers in non-covalent AP-encapsulation, by presenting existing formulation strategies and proposing new ones in order to improve performance. To this end, we will first identify the most important factors influencing the efficacy of dendrimer-based AP-nanovectors, and then we will describe some possible strategies, both experimental and theoretical, to optimize their further development and application.

2. Current state-of-the-art: Main factors involved in non-covalent drug association to dendrimers

Historically, the theoretical concept of the physical uptake of small molecules by highly branched macromolecular architectures was suggested first by M. Maciejewski in 1982 [50]. The first publication reporting the use of dendrimers in APs formulation (*i.e.*, of aspirin with poly(amidoamine) (PAMAM) dendrimers) dates back to 1989 [51]. Since then, the successful encapsulation of dozens of different APs by dendrimers has been reported, and the number of studies focused on this topic continues to grow. The impressive amount of accumulated data permits analysis of the most important theoretical and practical aspects of the use of dendrimers for this purpose. With this in mind, the following section of this review will cover the main factors, phenomena, and mechanisms involved in this AP-vectorization approach, including the mechanisms of non-covalent dendrimer-AP association, dendrimer-dendrimer interactions, as well as biological properties relevant to the host dendrimers.

2.1. Dendrimer-drug interactions

2.1.1. Mechanisms of non-covalent dendrimer-AP association

An understanding of the function of dendrimers as AP-encapsulating agents can be approached by an effective combination of physico-chemical interactions such as electrostatic forces, hydrophobicity, π-π stacking, hydrogen bonds, and the effects of physical steric immobilization [52–55], none of which lead to the formation of covalent bonds with the AP. The presence of these interactions is determined by the chemical structure of the dendritic skeleton and the AP. Conceptually, an optimal combination of dendrimer's structural features should facilitate the attraction of the AP-molecules at the dendrimer's

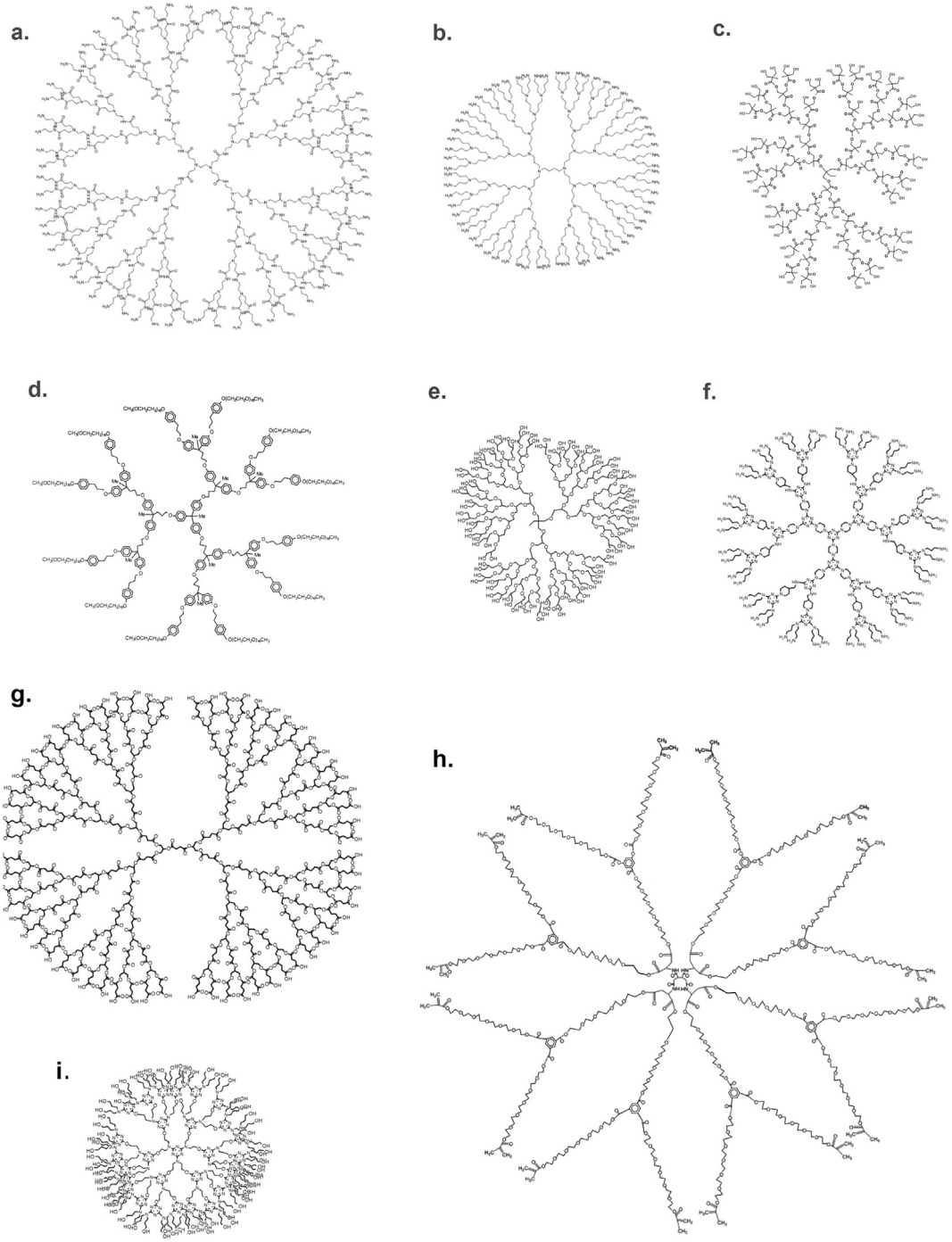

Fig. 2. Examples of different dendrimer families proposed as non-covalent AP-encapsulating agents: (a) poly(amidoamine) dendrimer (PAMAM) elaborated by D. Tomalia [6]; (b) poly (propyleneimine) dendrimer (PPI) obtained independently by research groups of E. W. Meijer and R. Mulhaupt [6]; (c) idealized structure of commercially available pseudo-dendrimer *Boltorn H40* assembled from BMPA [107]; (d) dendrimer with the interior composed of *p*-phenylene aromatic rings linked by aliphatic short chains and the methoxy-terminated PEG-periphery, elaborated by M. Liu et al. [92]; (e) polyglycerol dendrimer synthesized by T. Ooya et al. [41]; (f) dendrimer assembled from melamine, piperazine and piperidine units, developed by the group of E. Simanek [96]; (g) dendrimer composed of glycerol and succinic acid units, reported by M. Morgan et al. [48]; (h) polyester-*co*-polyether (PEPE) dendrimer elaborated by R. Dhanikula et al. [98], combining different hydrophilic molecular segments, including PEG, DHBA, BTCA and aspartic acid; (i) dendrimer composed of triazine and diethanolamine units, elaborated by K. Bansal et al. [104].

periphery, then promote their retention inside the macromolecular matrix and ultimately allow releasing them under appropriate conditions, acting as a fine-controllable molecular container.

2.1.1.1. Mechanisms of AP-encapsulation by PAMAM, PPI dendrimers and their close derivatives. The most-studied dendritic structures proposed as AP-nanoencapsulation agents are mainly represented by poly (amidoamine) and poly(propyleneimine) dendrimers (PAMAM, PPI; Fig. 2a and b, respectively) and their close derivatives. This is primarily due to the relatively facile chemical synthesis conditions, the long-standing commercial availability of PAMAM and PPI, and to the possibility of modifying their peripheral primary amine groups, in order to change surface properties, if needed. The skeletons of PAMAM and PPI contain aliphatic primary and tertiary amine functional groups, which can foster the entrapment of APs through electrostatic (or ionic) forces, hydrogen bonds, or hydrophobic interactions [15,52,56,57],

among which the energy of interaction *via* ionic forces is generally the highest [58,80]. Consequently, the presence of positively charged amino groups in the dendritic structures and of negatively charged ones in the structures of the APs can greatly facilitate the process of dendrimer-AP association. Examples of successful formulation of acidic APs with PAMAM have been extensively reported, including with methotrexate (MTX) [37], chlorambucil [47], indometacin [59,60], ibuprofen [56], flurbiprofen [61], ketoprofen [62,63], diflunisal [63], enoxaparin [64], as well as mycophenolic [57], acetylsalicylic [51], nicotinic [65], benzoic and salicylic [66] acids, *etc.* In these cases, the encapsulation occurs in a two-step process, involving first the dendrimer's surface primary amines, and subsequently the inner tertiary ones [15,52,56,57]. The higher the G-number of the dendrimers, the stronger the electrostatic interactions which result in higher AP-encapsulation rates [68]. In this context, even bulky negatively charged siRNA and DNA have been effectively encapsulated in dendrimers of this type, including asymmetric PAMAM with supplementary polymeric chains grafted to the central core segment [67], as well as hybrid pseudo-dendritic structures of 5 to 25 kDa assembled by grafting PPI and PAMAM dendrons to a central core represented by the polyethyleneimine dendrimer (PEI, analogue of PPI) of pseudo-G3 [69]. Noteworthy here is that the encapsulation processes based on acid-base interactions are very sensitive to changes in pH. PAMAM and PPI dendrimers are evenly suitable for encapsulating non-acidic APs, as demonstrated with camptothecin [68], piroxicam [70], nifedipine [71], sulfamethoxazole [72], phenobarbital [73]. As in the above case of acidic APs, AP-encapsulation rates tend to increase with the G-number, suggesting that non-ionic interactions, such as hydrophobic attraction, steric immobilization, hydrogen bonds and dipole-dipole effects (van der Waals interactions), can also play an important role in AP-dendrimer complexation [68,70–73].

The AP-dendrimer-association mechanisms are influenced by chemical modification of the PAMAM and PPI periphery. In particular, the presence of peripheral hydroxy groups favours the formation of hydrogen bonds [15,16,44,66,74]. The peripheral esters, depending on the structures of organic substituents, tend to support hydrophobic interactions [15,71,75], while the carboxylic groups maintain interionic effects (*i.e.*, with basic APs [74]). The grafting of poly(ethylene glycol) (PEG) chains provides an increase in the solubility in aqueous media and enables improved biological properties (see more in Section 2.3.1). The PEGylation can also lead to an increase in AP-payload, compared to the unmodified macromolecules, as shown for MTX and doxorubicin (DOX) in the case of PAMAM G3 et G4 [76], and for 5-fluorouracile in the case of PAMAM G5 [46]. It should however be noted that the AP-encapsulation rates are not directly correlated to the PEG length. Several comparative studies have demonstrated an increase in drug loading from PEG550 to PEG2000 for PAMAM G3-G7 [76,77], while the use of bulkier PEG5000 leads to the opposite effect compared to PEG2000 [77,78]. In the last case, two possible mechanisms were proposed. The first one considers the possibility of agglomerates formation *via* the penetration of PEG5000 chains inside neighbouring dendrimers, thereby reducing the overall volume of the internal cavities available for encapsulation [78]. The second one, evidenced by *in silico* simulations, involves the self-folding of PEG5000-chain towards the dendrimer core [77]. Therefore, it can be reasonably expected that the critical length of peripheral PEG-chains for the AP-encapsulation efficiency will be strongly limited to the molecular weights of 1000–5000 Da, a more accurate value of which is still to be clarified. The use of less common bulky substituents such as poly(*N,N*-dimethylaminoethyl methacrylate) (DPMA) chains, allowed to decrease the release rate of chlorambucil compared to non-substituted PAMAM G3, even under partial modification of the dendrimer periphery [47]. Similar results were observed with DOX and DOX-pRF-hTRAIL conjugate encapsulated in PAMAM, the peripheries of which were modified with polysaccharides, (60-kDa dextran) [39], and with the peptides RGD (arginine-glycine-aspartic acid peptide) [79] and HAIYPRH (T7, transferrin receptor specific ligand) [80,81], respectively. The observed effects were explained by the increase in volume of the resulting dendrimers [39] without, however, proposing any detailed PA-retention mechanisms. The grafting of much less bulky molecules of mannose (up to 33) onto a PPI G5 led to a moderate encapsulation of lamivudine (8%) and to a significant 6-folds decrease in release rate, due to the increase in the number of groups available for complexation and the steric hindrance preventing the AP from leaving the dendrimer capsule freely [82]. In the same line, folic acid (FA) molecules at the periphery of PAMAM G4 [43,60], hydroxylated PAMAM G5 [16], and PPI G5 dendrimers [40] led to a slower release of indomethacin, 5-FU, MTX and DOX, respectively, through the increased hydrophobic interactions and steric effects [60]. Improved sustained release was observed with 5-FU when FA residues were grafted on PAMAM G4 *via* PEG4000-spacers [43], suggesting that in some cases, combinations of molecular segments with different physicochemical properties can be beneficial. At the same time, an attempt to attach *N-tert*-butoxycarbonyl (*t*-BOC) functions using a PEG5000 spacer on PAMAM G4 dendrimers had very little impact on the MTX release parameters, even by varying the number of *t*-Boc-NH-PEG5000-NHS units [37]. Especially noteworthy, the prevention of uncontrolled AP-release by steric hindrance maximization at the dendrimer surface should not be considered universal for drug delivery. For example, when the PPI-G4 periphery was modified with phenyl and *t*-BOC groups, the release of Rose Bengal stain was only observed after hydrolysis at reflux in 12 N HCl [75], which is not realistic for therapeutic applications. To be effective, this strategy necessitates more elaborated approach, taking into account not only the relative size of the AP molecules and the cavities available in the dendritic architecture, but also opportunities to effectively manage the process of capture and release. One clever approach is to use dendrimers with a periphery bearing substituted azobenzene groups. Sensitive to UV light, pH and to some enzymes as well, the azobenzene units enable PPI [83–86] and PAMAM [87] to effectively release model anionic and hydrophobic molecules (*i.e.*, organic dyes such as eosin) *via* a *trans-cis* geometric isomerization that can be effected readily and reversibly with low intensity light. This release motif triggered by external stimuli represents a recent and emerging class of 'release-on-demand' encapsulation systems that can provide many advantages over traditional release dependent on thermodynamic equilibrium shifts.

2.1.1.2. Mechanisms of AP-encapsulation by other types of dendrimers. Dendritic macromolecules other than PAMAM and PPI represent a very large and heterogeneous structural family (just the number of known polyester dendrimers alone exceeds several thousand [88]). We will therefore only provide a general overview of existing architectures as well as highlight the most important structural aspects directly related to drug delivery. Of note, some of these architectures were developed long before PAMAM and PPI, *i.e.*, the dendrimers of F. Vögtle reported in 1978 [4] and those of R. Denkewalter in 1981 [89]. Nevertheless, given that these dendrimers generally are not readily available, their experimental use as AP-encapsulating agents dates back only after 2000 and is still quite limited [90,91]. Recent years have seen the appearance of commercially available products at relatively large scale (more discussion below) providing hope of more development opportunities. In this section, more attention will be paid to the internal macromolecular structure of dendrimers, given that the role of peripheral functional groups in AP-encapsulation has been detailed in the previous section. The internal structure of dendrimers governs the size of inner cavities available to accommodate AP-molecules, as well as the types of physicochemical interactions with them, so can be used to rationally tailor and tune AP-specific loading and release.

In 2000, M. Liu et al. [92] reported the encapsulation of indomethacin by a G3 dendrimer, whose interior was composed of *p*-phenylene aromatic rings linked by aliphatic short chains and the periphery was decorated with hydrophilic chains of methoxy-

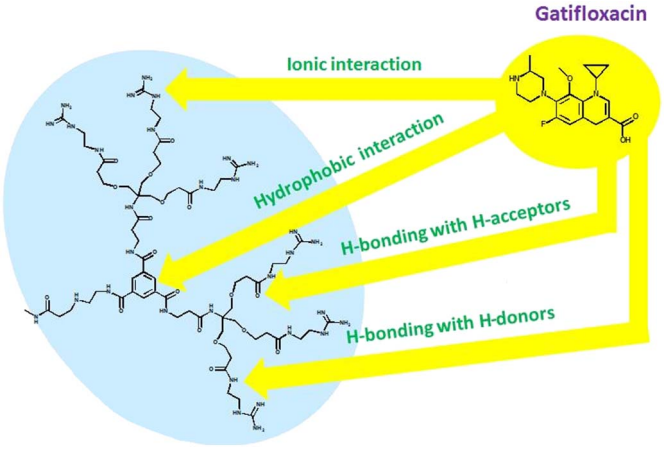

Fig. 3. Schematic representing the available sites for various physico-chemical interactions between the Durairaj dendrimer and gatifloxacin. Adapted with permission from Durairaj et al., Invest. Ophth. Vis. Sci. 51 (2010) 5804–5816 [105]. Copyright 2017 IOVS Online by the Association for Research in Vision and Ophthalmology.

terminated PEG700 (Fig. 2d). The authors observed slower *in vitro* release profiles *versus* the naked drug, which was explained as arising from strong hydrophobic interactions between the dendrimer interior and the AP [92]. In 2003, T. Ooya et al. [41] studied the solubilization of paclitaxel (PTX) in 10 and 80-% aqueous solutions of polyglycerols dendrimers of G3, G4 and G5 (Fig. 2e), reaching a 10^2–10^4-fold increase in PTX-solubility, compared to the naked AP, and a nearly 10-fold increase *versus* formulations with linear PEG400 and PEG2000. The improved effects were interpreted as a direct consequence of the higher density of ethylene glycol units in the dendritic architectures [41]. A year later, using NMR data, the same authors showed that the polyglycerol dendrimers were able to interact with the aromatic rings and methylene groups of PTX, thereby suggesting that the presence of hydrophobic segments in the dendrimers is not a determining factor to act as effective solubilizing agents of hydrophobic molecules [42]. Interestingly, the polyglycerol dendrimer of G3 was found to transform the lactam structure of 5-fluorouracil (5-FU) into its lactim form [93], illustrating the possibility of chemical transformations induced by the *a priori* "inert" structure. Further to this, the entrapment of the hydrophobic APs diclofenac, 5-aminosalicylic and mefenamic acid, by highly polar polyester dendrimers of G1–3 composed of linear PEG600-core and two dendrons formed by polycitric acid was reported by H. Namazi and M. Adeli [94]. The increase in AP-payload with each increased dendritic generation was linked with steric effects and dipole-dipole interactions. These dendrimers were also proposed as nanovectors for cisplatin by complexation of APs with polyanionic surface [95].

Another family of effective dendrimers has been developed by Simanek, based on melamine, piperazine and piperidine units (Fig. 2f) [96]. The high encapsulation rates of methotrexate and 6-mercaptopurine in these dendrimers were probably due to the presence of aromatic rings and polar and ionizable groups. M. Morgan et al. reported the encapsulation of hydrophobic camptothecins in a dendrimer of G4.5 assembled from natural metabolites, glycerol and succinic acid (Fig. 2g). Despite the 10-fold increase in solubility of the APs in water, the system showed a very rapid drug release in PBS buffer (up to 80% after 2 h), indicating the insufficient AP-dendrimer affinity for long-circulating formulations [48]. D. Bhadra et al. [97] carried out the encapsulation of chloroquine phosphate into dendrimers exhibiting a PEG1500- or PEG4000-core and poly-L-lysine dendrons. Both the AP-loading and the AP-release time increased with increasing dendrimer generation (G4 to G5), probably due to both steric effects and complexation affinity. Dhanikula et al. [98] effectively combined different hydrophilic molecular segments to obtain novel polyester-*co*-polyether (PEPE) dendrimers (Fig. 2h) capable of encapsulating hydrophobic β-carotene and rhodamine. The observed relatively slow and sustained release of the payloads (~90% after 120 h) was mostly explained by steric effects caused by the dendritic structures, due to the absence of π-π interactions, as shown by UV-spectral studies [98]. Different dendrimers of this family were employed to encapsulate MTX. The MTX-loading rates were found to increase with the PEG length and presence of aromatic branching points by both hydrophobic and aromatic interactions [35]. The AP-release profile in PBS buffer could be divided into two clearly distinguishable phases: firstly, a strong 'burst release' over 6 h, and secondly a sustained release extending for 170 h. Of note here, no significant change in drug encapsulation and retention efficiency was observed after modifying the dendrimer's peripheries by glycosamination [36,99]. Other PEPE families obtained recently by Hildgen's group were specially designed to encapsulate itraconazole (ITZ), a highly hydrophobic antifungal agent [100–102]. These structures differ by their central parts formed by organic residues possessing different rigidity while having the same *tetra*(ethylene glycol)-based periphery [101,102]. Encapsulation studies of ITZ including both *in silico* simulations and *in vitro* testing revealed an increase in AP-loading when the central part of the dendrimers was composed of more flexible hydrophobic segments [102]. The effect was explained by the possibility of effective bending of dendrimer segments around AP molecules, facilitating the retention by steric effects. For example, *O*-acylated glyceryl tris[9,10-(*threo*)-dihydroxyoctadecanoate] with three free alkyl chains was the most efficient, despite the absence of dipole-dipole or aromatic staking effects presented by other more rigid core structures [102]. Remarkably, similar conclusions about the importance of including flexible central segments in unimolecular nanocapsules were recently drawn by Lukowiak and al [103]. Particularly, the encapsulation rates of hydrophobic molecules were higher in hybrid core-shell nanoparticles with a more flexible polyethylene-dendrimer core *versus* a core represented by nano-diamond. Another family of dendrimers composed of triazine cycles and diethanolamine spacers (Fig. 2i), proposed by Bansal et al. [104], increased by 100 times the solubility of PTX in water as well as allowed control over its release time by hydrophobic interactions. In parallel, dendrimers having a 1,3,5-tricarboxybenzoïc-acid core and dendrons assembled from tris (hydroxymethyl)-aminomethane (TRIS), 3-hydroxypropionic acid, ethylene diamine and guanidine (Fig. 3) were elaborated by Durairaj et al. [105] as ophthalmic surfactants increasing the bioavailability of gatifloxacin. The mechanisms of interaction between the AP and the dendrimers were studied by FTIR, ^{1}H NMR and isothermal titration calorimetry (ITC), allowing the identification of ionic, hydrophobic and hydrogen-bond binding sites [105] (Fig. 3). A number of recent dendrimer architectures can be defined as new combinations of structural elements which are already well-known in dendrimer chemistry. Such is the case of dendrimers composed of pentaerythritol, 1-thioglycerol, BMPA et PEG, elaborated by Ma et al. for encapsulating DOX [106]. In this context, it is worth mentioning that systematic comparative studies aimed to identifying the most efficient arrangements for the macromolecules assembled from the same molecular residues are still, however, very limited.

In the framework of non-covalent drug-vectorization, some pseudo-dendritic architectures ('imperfect'- or pseudo-dendrimers) also deserve to be mentioned. Conceived as regular dendrimers, pseudo-dendrimers are mixtures of macromolecular homologues synthesized using the same initial monomers. Their 'imperfection' characterized by lower skeleton symmetry stems mostly from the incomplete chemical transformation of peripheral groups as well as from limitations inherent in purifying macromolecular products by common methods. Even so, the possibility of avoiding complicated and very expensive steps of purification required to obtain perfect dendrimer structures can offer a cost-effective option (often the decisive factor) when moving from small- to large-scale manufacturing. In this regard, recently, a Swedish company *Polymer Factory*[107] proposed a few series of different pseudo-dendritic products, such as polyesters *Boltorn*® and polyester-

polyamides *Hybrane®*, and polyamide-polyamine Helux®. In terms of AP encapsulation, the most interesting candidates are BMPA-based *Boltorn®* (more on the biological characteristics of polyester macromolecules to follow in Section 2.3), whose physicochemical properties were deeply studied by E. Zagar, M. Zigon [108,109] and U. Domanska et al. [110]. These pseudo-dendrimers differ in the molecular weight and nature of the groups grafted to the periphery. For example, *Boltorn H40* (of ~7.3 kDa) is a pseudo-G4-dendrimer, assembled only from BMPA, *e.g.*, Fig. 2c (idealized structure), while *Boltorn H2004* (~3200 Da), *Boltorn U3000* (~6500 Da) and *Boltorn W3000* (~9000 Da) are BMPA-based architectures obtained by surface post-modification with C6– and C14-chain fatty acids, and PEG chains, respectively. R. Reul et al. [111] reported the encapsulation of PTX by *Boltorn H40*, *U3000* and *W3000*. The presence of fatty acids on the surface of *Boltorn H40* and *U3000* led to a higher AP-payload due to the possibility of additional hydrophobic interactions with PTX. Interestingly, the AP-release profiles showed no difference between the samples. In addition, the presence of 70–170-nm particles in solution indicates the formation of supramolecular systems. *Boltorn H40* can also be used as a starting reagent in syntheses of other hyperbranched architectures, *i.e.*, *via* their polyhydroxyl periphery modification. For example, S. Aryal et al. [112] applied the grafting of poly(ε-caprolactone) obtained *in situ*, using *Boltorn H40* as a multifunctional initiator, followed by the esterification of terminal hydroxyl groups with MeO-PEG2000-COOH. The product was tested as an encapsulating agent for 5-FU, demonstrating a 26-% AP-payload. In PBS buffer, the 'burst release' of 5-FU (up to 40% of the initial charge after 8 h) was observed, followed by a sustained liberation for about 130 h [112]. X. Zeng et al. [113] reported the encapsulation of DOX, using *Boltorn H30* and *Boltorn H40* modified with PEG5000 and PEG10000. Longer PEG chains tended to reduce AP-loading (from 4 to 3%), which is in accordance with conclusions drawn in Section 2.1.1.1. Nevertheless, the AP-release in PBS buffer was more prolonged in the case of the bulkier PEG10000-perifery. Of note here, formulating with DOX resulted in 6-to-16-fold increase in the particle size, indicating aggregation, and consequently the possibility of an additional PA-release mechanism, not only from the internal cavities (see also Section 2.2).

2.1.2. Influence of medium properties on the stability of AP-dendrimer complexes

In addition to the structural characteristics of dendrimers and APs, the process of formation of complexes between them may also be greatly affected by the properties of the dispersion medium (*i.e.*, pH, t °C, presence of electrolytes, proteins, *etc.*). The sensitivity to pH is generally determined by the presence of ionizable acidic or basic functional groups [15]. In this case, the pH may favour interactions of dendrimers either with APs or with the external medium, by firming or destabilizing complexes, respectively. For example, the stability of systems composed of polycationic dendrimers (PAMAM and PPI) and acidic APs (*i.e.*, MTX and all-trans-retinoic acid [49], chlorambucil [47], nifedipine [71], flurbiprofen [61], indomethacin [114]) is higher in neutral and/or basic conditions than in acidic environments, confirming that the AP-encapsulation is chiefly induced by ionic forces. Similar effects were observed with modified PAMAM and PPI exhibiting non-ionic acetylated [71], azobenzene [83] or FA [40] functional groups at their peripheries, involving the internal tertiary amine functions of dendrimers. The higher stability of the inclusion complexes in neutral and basic media is also observed when APs are basic (*e.g.*, famotidine [114]) and amphoteric (*e.g.*, amphotericin B [114]) molecules, suggesting that both, polycationic dendrimers and APs, are associated by non-ionic mechanisms, *i.e.*, hydrophobic and van der Waals forces. In the case of non-ionisable dendrimers, the sensitivity to pH hinges on the possibility of AP-ionization, as, for example, shown for the basic DOX encapsulated in PEPE architectures [106]. Temperature is another important factor influencing dendrimer-AP association. PE-Gylated and polyamino-PAMAM dendrimers with ibuprofen [56], 5-FU [46] or piroxicam [70] payloads, showed a significant AP-loss and the occurrence of precipitation and degradation products with the rise of temperature to 50–60 °C. Furthermore, the AP-loss was much higher at simultaneous light exposure [46,70]. The necessity of taking into consideration the presence of electrolytes is supported by the fact that a faster AP-release in buffered saline is often observed *versus* that for simple aqueous solutions [15]. For example, at pH 7.4, PAMAM G3–G4 modified with MeO-PEG550 and MeO-PEG 2000 at their peripheries demonstrated a more rapid release of MTX in isotonic 150 mM NaCl compared to 1 mM TRIS-HCl buffer. This is due to weaker ionic interactions between dendrimers and AP with the increasing concentration of electrolytes [76]. The multifunctional structure of plasma proteins can also interfere with the AP-dendrimer association process, leading to non-targeted or early AP-release [15,57]. The presence of albumin even at 1% concentration causes the accelerated release of a piroxicam payload encapsulated into PAMAM G3–4, compared to that in protein-free buffer [70]. Fluorescence measurements revealed that the interactions of PAMAM G3.5–4.0 having $-NH_2$, –OH and –COOH peripheral groups with negatively charged albumin are mostly limited to strong electrostatic effects and can induce conformational changes (denaturation) of the protein [115]. In addition, APs can demonstrate more affinity for albumin than for the vectors, probably, due to the better arrangement of molecular segments responsible for electrostatic and hydrophobic interactions, as shown with 1-anilinonaphthalene-8-sulfonate and PAMAM [116]. Similar results were obtained after adding serum to solutions of PTX encapsulated in polyglycerol dendrimers [41]. Thus, the above outcomes are consistent with data obtained *in vivo* demonstrating the significantly higher AP-release after administration to animal models *versus* simple aqueous solutions. Despite evident progress, the exact mechanisms of AP-release *in vivo* are still poorly understood, given that the *in vivo* environments represent very complex and dynamic media, which are extremely difficult, if not impossible, to model and study accurately. In addition to the above factors, changes in dendrimer's chemical structure induced by biological agents should also be considered (see more on bio-degradable dendrimers in Section 2.3.1.2).

2.2. Dendrimer-dendrimer interactions

In most studies related to dendrimer-based nano-vectors, dendrimer-dendrimer interactions are largely overlooked, yet are often quite relevant and important. One of the after-effects of such interactions would however be readily detectable; in particular, it is a question of a spontaneous increase in size of dendrimer particles in solution due to direct dendrimer-dendrimer aggregation. The aggregation processes can, in turn, alter important formulation parameters such as the AP-encapsulation rate, the AP-release profile, size-dependent bio-distribution and bio-absorption. As a matter of fact, such dendrimer-based aggregates should not normally be considered as unimolecular encapsulation systems [117], and therefore, supplementary studies should be undertaken to establish the optimal particle size distribution and mechanisms of AP-encapsulation/release.

In this context, it is interesting to note that to date, there is no experimental evidence supporting the concept of the 'unimolecular behaviour' of dendrimers in aqueous media. In the large majority of the reported cases, *e.g.*, [45,98,111,113,118–120], results of the size measurements (by, *e.g.*, DLS, SEM and AFM techniques), indicate the presence of dendrimer aggregates in suspension rather than unimolecular solutions (Fig. 4). Generally, it is reasonable to expect that the aggregation mechanisms depend on surface properties of dendrimers and involve similar interactions to those discussed in Section 2.1. The most recent attempts to study the aggregation mechanisms using *in silico* modeling showed that the attraction between macromolecules is due to van der Waals interactions between uncharged regions of the dendrimer surfaces [121]. Therefore, it is reasonable to assert that 'unimolecularity' in aqueous solutions as a distinctive property of dendrimers

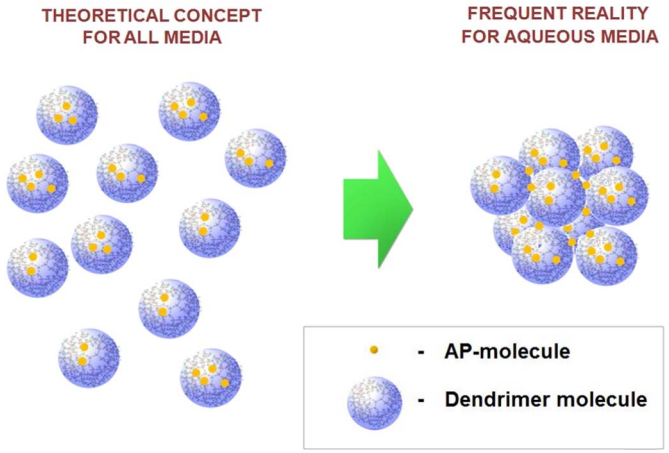

Fig. 4. Schematic representation of the theoretical concept of dendrimer unimolecular behaviour in solution proposed for all media and the formation of dendrimers aggregates frequently observed in real aqueous media.

can be achieved only at a sufficiently high surface charge density, which obviously depends on the nature of the terminal groups and the G value, as well as on the parameters of the media and the dendrimer concentration. At this point however, the optimal surface properties are still to be clarified, since the aggregating behaviour has been observed not only for highly hydrophilic noncharged peripheries composed, for *e.g.*, of PEG groups [35,98,101,102,112], but also for the case of highly charged COONa-surfaces [122]. In particular, in the last example [122], a preliminary filtration of dendrimer solutions was not sufficient to prevent the presence of aggregate particles, the size of which was exceeding the filter porosity, suggesting that the aggregation process was spontaneous and energetically favourable. Dendrimer-dendrimer interactions can also be affected by the presence of AP-molecules. The increase in size of particles formed by AP-dendrimer complexes [45,98,101,102,111,113,118–120] could be explained by the 'bridging effect' exerted by the AP, as demonstrated by *in silico* studies on model systems [123]. Consequently, the concept of dendrimer-based-nanovectors proposed as 'unimolecular nano-boxes' must be carefully used and demands strong experimental evidence.

Instead of avoiding the aggregation of dendrimers, several researchers have proposed to take advantage of it to form regular supramolecular assemblies. Giles et al. showed that a guest molecule could be used as a template at neutral pH to generate a trimolecular inclusion complex, by testing a large range of hydrophobic guest substances (Fig. 5), including biologically active estradiol [124]. Additionally, recent data have shown the possibility of obtaining bilayer spherical structures (dendrosomes) of 2–50 μm in size, composed solely of dendritic molecules [125]. A promising avenue of dendrimer development is the design of non-symmetric branched architectures presenting amphiphilic properties, leading to the formation of multilayered polyfunctional dendrimeric polyplex micelles for overcoming various physiological barriers [126], or light-induced gene transfer [127,128]. Such an approach could open up new perspectives in developing novel submicronic dendritic AP-vectors benefiting from their supramolecular behaviour.

2.3. Biological properties of dendrimers

2.3.1. Bio-distribution and biosafety of dendrimers

The use of dendrimers as AP-delivery vectors requires one to consider the interactions of these macromolecules with the organism. This should take into account the factors which include not only the potential therapeutic benefits of the formulation but also the potential biological risks that future patients could run. In this context, biological properties of dendrimers have been intensively studied over the last few decades [129,130] and can be briefly summarized into two main characteristics, namely, bio-distribution and biosafety, the main aspects of which will be discussed below in detail.

2.3.1.1. Bio-distribution. The bio-distribution of dendrimers is determined by their surface properties, such as the size, the surface charge, and the presence of hydrophilic and/or hydrophobic groups. Polycationic dendrimer peripheries (*i.e.*, due to the ionized amino groups) are known to interact by strong electrostatic effects with anionic functional groups of cell membranes [15]. An effective use of dendrimers as AP-vectors for oral administration, except a few cases [29,38,141,146], is still limited, probably, due to many biological barriers to overcome. After intravenous (*iv*) or intraperitoneal (*ip*) administration in mice, polycationic PAMAM and poly-lysines, tend to be rapidly absorbed at the surface of blood vessels, that prevents their effective bio-distribution throughout the body [131–133]. Depending on the G-value and animal model, the polyamine PAMAM, poly-lysines and polymelamine dendrimers can distribute differently in the liver, kidneys and pancreas [131,133,134]. The modification of the dendrimer surface with anionic (acidic) groups can increase their residence time in the bloodstream, as reported for polycarboxylated PAMAM [133]. The nature of the surface acidic groups can also impact the bio-distribution. Poly-lysine dendrimers with polysulfonate peripheries were opsonised quickly and retained mainly by the reticuloendothelial system (liver and spleen), while dendrimers bearing succinate groups were rapidly eliminated by renal clearance [135]. Non-ionized hydrophilic polyhydroxy terminated PAMAM provide site-specific delivery of small molecular drugs across the blood-brain and blood-cerebrospinal-fluid barriers (BBB and BCSF, respectively), specifically targeting the injured brain in canine [134] and rabbit [136] models. In rabbit models, polyxydroxylated PAMAM are also found to be more effective in crossing the fetal blood-brain barrier, and targeting fetus activated microglia than polycarboxylated PAMAM [137]. The bio-distribution studies in rats with PEGylated PAMAM and poly-lysine dendrimers demonstrated a marked increase of the bloodstream residence time and a significant lowering of the dendrimer accumulation in the liver, a decrease in elimination by renal filtration and an increase in reaching the lymphatic system for

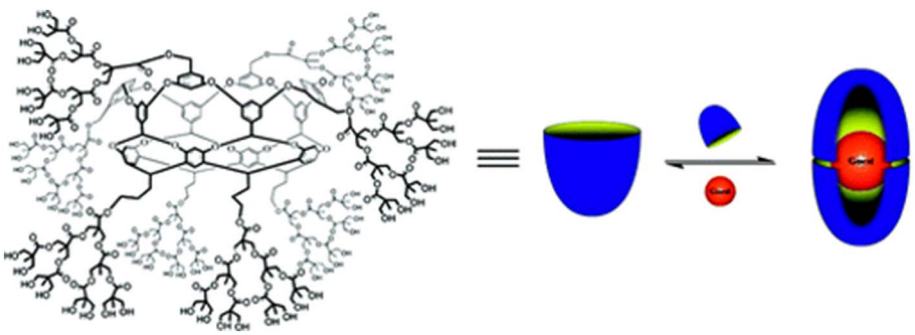

Fig. 5. Formation of a nanocapsule from two dendrimers, each carrying an internal hydrophobic hemisphere, around a hydrophobic molecule in water at neutral pH. Reprinted with permission from Giles et al., J. Am. Chem. Soc. 130 (2008) 14430–14431 [124]. Copyright 2017 American Chemical Society.

structures having higher molecular weights [133,138–140]. Similar conclusions were drawn for the dextran conjugated PPI G5 [39]. Interestingly, the presence of the peripheral hydrophobic tetradecane groups may impart a moderate increase in AP-concentration in the bloodstream after oral administration [141]. Coating dendrimers with phospholipids may also increase the uptake by the lymphatic system [119]. The ^{125}I–labeled non-ionized polyester dendritic scaffolds (up to 23 kDa) presenting different arrangements of BMPA and PEG showed that the rapid excretion in urine after *iv* administration in mice is mostly dependent on the molecular weight of the macromolecules and not on their degree of ramification [142]. On the other hand, the larger dendrimers (40 kDa and greater) with a more pronounced branched architecture, demonstrated an increase in circulation time and a decrease in renal clearance, probably due to their lower flexibility and consequently, to the difficulties in passing through glomerular pores [143]. Remarkably, no specific organ accumulation was observed for these macromolecules, despite a significant portion of the dose found in the carcass after 48 h [143].

In the context of bio-distribution, it is worth mentioning *ex vivo* and *in vitro* studies dealing with the interactions of dendrimers with biological membranes at the tissue and cell levels respectively. The *ex vivo* studies are mostly limited to the general membrane penetration tests, in some cases, demonstrating selectivity to specific tissues, depending on the dendrimer structure. For example, the extravasation duration of polycationic PAMAM G0–4 labeled with fluorescein isothiocyanate through the microvascular endothelium in hamster cremaster muscles increases with G [144]. Concomitantly, PAMAM G4-NH_2 demonstrated only very limited transfer rates to the fetus side of human placenta [145]. The ^{125}I–labeled polyanionic PAMAM (G 2.5, 3.5, and 5.5, − COONa) showed a better penetration capacity through the intestinal sac of adult rat compared to dendrimers with polycationic periphery (G3 and G4, − NH_2) [146]. In porcine ear skin, the infiltration enhancement of PAMAM with different peripheral functionalization (− COOH, –OH, − NH_2) was found to follow: G4-NH_2 > G4-OH > G3.5-COOH. Skin penetration resistance was also found to increase with G for G2-G6-NH_2[44]. Similar results were observed in an *in vivo* study on rabbit eye corneas, using PAMAM G2- and G4-NH_2, G2- and G4-OH, G1.5- and G3.5-COOH [74].

The dendrimer surface charge and area also play an important role in interactions between dendrimers and cell walls membranes, as demonstrated by *in vitro* assays on different cell lines. In particular, the enhanced cell membrane permeability observed with dendrimers having polycationic peripheries can be associated with their capability to non-selectively disrupt the negatively charged cell walls (in some cases, creating pores of 15–40 nm [147]), as shown on the human corneal epithelial (HCEC) [105], human epidermoid carcinoma (KB) [147], rodent fibroblast Rat-2 [147] and L929 [148], heterogeneous human epithelial colorectal adenocarcinoma (Caco2) [149], human umbilical vein endothelial (HUVEC) [150] cells, *etc.* Accordingly, the absence of positively charged surface groups considerably restrains interactions of dendrimers with the cell membrane. For example, the fluorescently labeled PPI and PAMAM with neutral polyacetyl or PEG peripheries showed a substantial loss of polymer adhesion to the plasma membrane and then damage, even at longer incubation times (> 60 min), compared to their polyamino precursors [147,150]. There is no association between the polyacetilated PAMAM dendrimers and the folate down-regulated KB cells [151]. Analogously, a pronounced decrease in transfection efficiency was observed after transforming PAMAM G4-NH_2 into its G4-OH derivative, suggesting that the polyhydroxy structure is significantly less capable of binding electrostatically to the surface cell-matrix and cell-cell anchoring proteins, such as heparan sulfate proteoglycans [152]. It should however be noted that in the absence of electrostatic effects, other dendrimer-transfection mechanisms are also possible. For example, energy-dependent fluid-phase endocytosis was evidenced by fluorescence microscopy in the case of *star*-shaped PEGs bearing polyesters of G1–4 [129]. Rhodamine-labeled PEPE dendrimers were found to be able to cross the cell membrane by both energy-dependent and clathrin- and caveolin-mediated endocytosis pathways [99].

The bio-distribution studies focused on exploring the 'active transport' approaches should also be mentioned. In this case, the surface of dendrimers is partially modified by grafting molecular ligands, in order to provide a high selectivity to the cells possessing the corresponding membrane receptors (*e.g.*, dendrimers decorated with a tripeptide arginine-glycine-aspartate showed an increased selectivity to cells with integrin receptors [153]). Although it is extremely difficult to find the receptor-ligand combinations to insure their targeting specificity, recent advances in this field have shown very encouraging results when treating some affected cells and pathogens (see more in Section 2.3.2). The intercellular fate of dendrimers is another important aspect of their biological properties, especially when it comes to specifying their influence on the host-cell machinery. This aspect represents one of the less studied areas of dendrimer properties and is mostly restricted to a few toxicological studies [129,154] (see more in Section 2.3.1.2). It is worth also mentioning the recent advances in creating artificial model systems as well as the virtual structures intended for computational (*in silico*) simulations. Given the tremendous challenge in modeling complex biological systems, such approaches are still limited to elucidating the molecular mechanisms driving the most simple intermolecular interactions. For example, a very good consistency was observed in testing the phospholipid membrane permeability by dendrimers with different surface properties between cell cultures studies, artificial phospholipid bilayer vesicles modeling, and *in silico* simulations [55,147]. *In silico* approaches gain more popularity when studying the physico-chemical interactions of dendrimers with genetic material (DNA and RNA) and proteins [55].

2.3.1.2. Biosafety. Overall, our knowledge of the biosafety of dendrimers needs to include possible adverse effects at the cellular, tissue, and organism levels after both short and long exposures [130]. For example, for any polymeric AP-vectors designed for parenteral administration, it is essential for the formulation to be non-toxic, non-immunogenic, and preferably, bio-degradable [129]. In other words, the biosafety analysis needs to include many multi-parameter tests *in vivo*, the implementation of which represents a tremendous challenge, given their complexity and high cost. Consequently, in the vast majority of cases, data on the biosafety of dendrimers in humans are still very poorly documented. For example, T. Toyama et al. [155] reported one case of toxic dermatitis developed in a Japanese student of 22 years of age, as a possible response of his immune system to the topical contact with a dendrimer. However, the exact nature of the substance that caused these effects has not been specified with certainty. In this regard, it should be noted that systematic *in vivo* toxicity tests in humans, using dendrimers for AP-delivery, are still nonexistent. This is probably due to the insufficient level of development in this area as well as to unsatisfactory results obtained during preliminary tests in cell cultures and animals. Therefore, so far, the general toxicological profile of dendrimers is mostly limited to data collected using different *in vivo* and *in vitro* model systems.

As a rule of thumb, dendrimers presenting polycationic groups should be expected to provoke increased toxicological effects. *In vivo* tests showed that after *ip* administration of polyamino PAMAM in mice, G7-NH_2 caused 40%-mortality, while G3-NH_2 and G5-NH_2 did not exhibit any short-term acute complications. In 7- and 30-day multiple dose experiments, the histopathology analysis revealed some vacuolization of cytoplasm in all liver samples [131] indicating possible lysosomal storage problems [129], however, no significant liver damage (assessed *via* the expression of pyruvic glutamic transaminase) was found after *iv* administration in mice of 10 mg/kg doses [139]. In addition, no evidence of immunogenicity was established, after administrating PAMAM-NH_2 in rabbits [131]. Similarly, other polyamino-terminated dendrimers, *i.e.*, composed of melamine [156] and lysine [140]

units, did not show hepatotoxic effects in mice at a 10 mg/kg *iv* dose, while the its increase to 40 mg/kg and 160 mg/kg dose led to an important hepatic necrosis and to the animal death, respectively [156]. On the other hand, dendrimers with neutral or acidic peripheries are found to be much better tolerated. For example, the PEG-grafting (typically of 0.2–40 kDa [157]) allowed the dendrimer tolerability to increase dramatically to the g/kg-dosing range, *i.e.*, up to 2.56 g/kg (*ip*) et 1.28 g/kg (*iv*) as demonstrated with the G2 polymelamine-piperazine-pyrimidine structures in mice [158]. No negative changes had been reported in the anatomy of mouse organs such as the liver, kidneys, spleen, heart and lungs after *iv*-administration of 10-mg/kg doses of a PEGylated polylisne G6 [140]. Blood parameters such as hemoglobin level, RBC, WBC, differentiated monocytes, lymphocytes and neutrophils, measured after 14 days following administration of PEGylated PAMAM-G4 (12 mg/kg) in rats, did not show significant changes compared to the control group [46]. No gross toxicity, either acutely or chronically up to 99 days (including a study for behavioural and histopathology abnormalities), was observed after a single 10 mg/kg *iv* injection of acetyl and folate PAMAM-G5 conjugates [159]. G3-triazine-based polyhydroxy (diethanolamine) terminated dendrimers showed no hemolytic effects and toxicity to the kidneys and the liver in mice up to 200 mg/kg *ip* injection [104]. No side effects were also reported in mice after repeated *ip* daily 95-mg/kg administration of PAMAM-G3.5-COONa [25]. A study on zebrafish embryos showed that PAMAM-G4-NH_2 affected the embryonic growth and development at sub-lethal concentrations, while PAMAM-G3.5-COONa was practically harmless [160].

Biosafety assessment performed *in vitro* on different cell lines also suggests that the nature of the surface groups influences the short-term toxicological profile of dendrimers. As discussed in Section 2.3.1.1, these groups are responsible for the capability of crossing the epithelial cell membrane, due to electrostatic attraction. Consequently, dendrimers with polycationic peripheries are able to disrupt the cell membrane integrity, affecting the cell viability. For example, dendrimers bearing primary amine groups exhibit hemolytic properties [46,61,70,133] associated with some important visually detectable changes in the morphology of erythrocytes (RBC), as well as with the release of hemoglobin induced by cell membrane leakage. Strong decreases in survival rates were also reported for many different cell lines, such as rat liver Clone-9 [156], HCEC [105], KB [147], rodent fibroblasts Rat-2 [147] and L929 [148], Caco2 [149], HUVEC [150], *etc.* It is noteworthy, however, that a comparative study [29] on L929 and RBC showed that dendrimers with a polyamine periphery (*i.e.*, PAMAM-G3-NH_2) were less toxic than linear polyamine polymers, in the following ranking: poly(ethylenimine) = poly(L-lysine) > poly(diallyl-dimethyl-ammonium chloride) > diethylaminoethyl-dextran > poly(vinyl pyridinium bromide) > PAMAM-G3-NH_2 > cationized albumin > native albumin. Also of importance, the mechanisms underlying the cytotoxicity effects caused by amino-terminated dendrimers are not limited to their interactions with the cell membrane. For example, the exposure of U-937 human macrophages to PPI G2-NH_2 and G3-NH_2 at concentrations > 90-% survival led to significant fluctuations of the reactive oxygen species (ROS) content and of the mitochondrial membrane potential, both related to the apoptotic activation of this organelle [161]. In addition, even without affecting the integrity of the cell membrane, dendrimers can dramatically affect the cell functions and provoke DNA cleavage [161], suggesting their very complex adverse impact on the cell machinery. Compared to this, dendrimers with neutral and/or negatively charged peripheries (*e.g.*, –OC(O)Me, − OMe, − PEG-OMe, –OH, –COOH, $-SO_3H$, $-PO(OH)_2$ are considerably less cytotoxic at short-term exposure, as reported in many cases [23,39,61,104,133,147,150–153,158,162]. The beneficial effect is generally explained by the decrease in capacity of interacting electrostatically with the external cell-membrane matrix (see in Section 2.3.1.1).

Dendrimers must also be safely transformed and/or removed from the organism to avoid long-term undesirable effects. In this connection, non-bio-degradable hydrophilic linear polymers that cannot be eliminated by renal clearance (*i.e.*, with Mw > 18 kDa) tend to accumulate inside the body, causing deposit formation in different organs, especially, in the skin and muscles [163]. The accumulation of dendrimers having relatively stable structures (PPI, PAMAM, poly-lysines, polyethers) mostly occurs in the liver [15,129]. In addition, the intracellular accumulation of non-bio-degradable macromolecules can cause lysosomal disease [129,164]. To avoid this problem, a promising strategy is to develop dendrimers containing bonds that can be broken over time under biologically relevant conditions, leading to the formation of non-toxic by-products. One of the most hopeful solutions is to employ polyester scaffolds that can be relatively easily degraded by carboxyesterases [15,165–167], as well as under weakly acidic or basic conditions [143,165]. Bio-degradability can also be achieved by inserting disulfide bridges sensitive to glutathione (GSH), a tripeptide produced by the majority of mammalian cells [15,28,168,169] (see also Section 2.3.2). The advantages of ester and disulfide groups can be effectively combined in the same structures, as carried out in a series of commercial dendrimers, PFD® [107]. Among other degradable macromolecules, some polycarbamate [170,171] and poly(phenethyl carbonate) [172] have also be reported, but a more thorough toxicological assessment is, however, still needed.

2.3.2. Proposals of encapsulating FDA-approved APs for specific therapies

A successful clinical use of dendrimers as non-covalent AP-encapsulating agents in specific therapeutic treatments would represent the quintessence of the significant basic work in materials development accomplished over preliminary stages. So far yet, no commercially available AP-formulations of this type are present on the pharmaceutical market, as well as no data on ongoing clinical trials with dendrimer excipients were publicly reported. At the same time, some dendrimer-based APs have already begun to gain recognition in medical applications, *e.g.*, Vivagel™ is now used topically in preventing and treating sexually transmitted viral and bacterial infections [173]. Another compound, a multiantigenic peptide PHSCN-lysine dendrimer, is currently undergoing preclinical study as a very potent inhibitor of human breast cancer cell invasion, extravasation, and lung colony formation [174]. Therefore, it is safe to assume that in the near future some promising dendrimer based excipients, *i.e.*, intended for AP-encapsulation and delivery (see Table 1), will be available for wide application.

2.3.2.1. Encapsulating APs for antineoplastic chemotherapy. The potential use of dendrimers as AP-vectors in antineoplastic chemotherapy has traditionally attracted more attention [181]. The increase in prevalence and the gravity of the disease (now the second leading cause of death globally, nearly 1 in 6 deaths, 8.8 million in 2015 [182]), as well as very costly treatments, are of strong interest to governments and private investors [181,183]. Accordingly, the ultimate (though still unreached [31]) goal of all drug vectorization strategies is to ensure a highly selective delivery to tumors of antineoplastic APs, which are mostly very toxic and attack all cells in rapid division, including healthy ones, by materially reducing beneficial effects. A delivery system should thus isolate the healthy environment from the APs (*i.e.*, by an effective encapsulation, which can also lead to a significant increase in solubility of highly hydrophobic APs [35–49]) and maximize its exposure to the target site.

Targeting of tumors is often based on the hypothesis of the prevailing role of the tumor blood vessel's bigger fenestrations which, together with the weakened function of lymphatic drainage, leads to the passive accumulation of nano- and submicron objects represented, *e.g.*, by dendrimers [7–14], in tumors over time (EPR effect, criticized recently as a conceptually inadequate prerequisite for selective AP-delivery in human [31]). One approach for targeting cancer cells takes advantage of the acidic pH values of tumor tissues which could be used as an effective internal trigger for releasing APs. The pH sensitive

Table 1
Proposals of using dendrimers as non-covalent FDA-approved-AP-encapsulating agents in specific therapies.

Aimed therapeutic activity	Encapsulated FDA-approved AP	Type of dendrimer structure	Type of test performed[a]
Antibacterial and antifungal	Amphotericin B [114]	Polyamino terminated PPI [114]	*In potus*[114]
	Benzoic acid [66]	Polyhydroxy (Tris) terminated PAMAM [66]	*In potus*[66]
	Gatifloxacin [105]	Polyguanidyl terminated dendrimers composed of guanidine, ethylenediamine, amides of 1,3,5-tricarboxybenzoïc and 2-hydroxypropanoïc acids [105]	*In potus*[105], *in vitro*[105], *in ex vivo*[105], *in vivo*[105]
	Itraconazole [101,102]	PEPE dendrimers composed of *O*-acylated glyceryl tris[9,10-(*threo*)-dihydroxyoctadecanoate] core, succinic acid spacers and PEG-peripheries [101,102]; PEPE dendrimers with tetra (ethylene oxyde) (TEG) peripheries, succinic acid, TEG and 1,10-decanediol spacers, and cores represented by pentaerythritol, tetraphenylporphine [102]	*In potus*[101,102], *in vitro*[101]
	Salicylic acid [66]	Polyhydroxy (Tris) terminated PAMAM [66]	*In potus*[66]
	Sulfamethoxazole [72,73]	Polyamino terminated PAMAM [72,73]	*In potus*[72,73], *in vitro*[72]
	Triclosan [175]	Polycarboxy terminated PAMAM [175]	*In potus*[175], *in vitro*[175], *in ex vivo*[175]
Antiepileptic (Sedation)	Phenobarbital [73]	Polyamino terminated PAMAM [73]	*In potus*[73]
Anthelmintic	Niclosamide [185]	Polyamino terminated PAMAM [185]	*In potus*[185]
Antihypertensive	Nifedipine [71]	Polyamino terminated PAMAM [71]	*In potus*[71]
Antiinflammatory and analgesic	Aspirin [51]	Methyl ester terminated PAMAM [51]	*In potus*[51]
	Cortisol [52]	Dendrimers composed of PPI dendritic core, TEG peripheries, urea and hexamethylene spacers [52]	*In potus*[52]
	Diflunisal [63]	Polyamino terminated PAMAM [63]	*In vivo*[63]
	Ibuprofen [56]	Polyamino terminated PAMAM [56]	*In potus*[56]
	Indomethacin [59,60,114]	Polyamino terminated PAMAM [59]; poly-FA terminated PAMAM [60]; polyamino terminated PPI [114]	*In potus*[59,60,114], *in vivo*[59,60]
	Flurbiprofen [61]	Polyamino terminated PAMAM [61]	*In potus*[61], *in vitro*[61], *in vivo*[61]
	Ketoprofen [62,63]	Polyamino terminated PAMAM [62,63]	*In potus*[62], *in vivo*[62,63]
	Mefenamic acid [186]	Polyamino terminated PAMAM [186]	*In silico*[186]
	Piroxicam [70,187]	Polyamino terminated PAMAM [70,187]	*In potus*[70], *in vitro*[70], *in vivo*[70], *in silico* [187]
	Sulfasalazine [188]	PPI with polyfucosylate periphery [188]	*In potus*[188], *in vitro*[188], *in vivo*[188]
Antimalarial	Artemether [176]	Polylisine dendrimers with PEG core having either polylisine or chondroitin peripheries [176]	*In potus*[176], *in vitro*[176], *in vivo*[176]
	Chloroquine phosphate [97]	Polylisine dendrimers with PEG core having either polylisine or chondroitin peripheries [97]	*In potus*[97], *in vitro*[97], *in vivo*[97]
	Primaquine phosphate [177]	Polyamino and polygalactose PPI [177]	*In potus*[177], *in vitro*[177], *in vivo*[177]
Antineoplastic	Camptothecins [48,68]	Polycarboxy terminated dendrimers composed of glycerol units as branching points and succinic acid core, spacers, and periphery [48]; polyamino terminated PAMAM [68]	*In potus*[48,68], *in vitro*[48]
	Carboplatin [178]	Polycarboxy terminated PAMAM [178]	*In vivo*[178]
	Chlorambucil [47]	Dendrimers composed of PAMAM core and PDMA periphery [47]	*In potus*[47]
	Cisplatin [25,95,179]	Polycarboxy terminated PAMAM [25]; PPI with diaminobutyrate periphery [179]; polyester dendrimers with polycitric acid periphery composed of PEG-core and polycitric acid dendrons [95]	*In potus*[25,179], *in vitro*[95], *in vivo*[25]
	DOX [38–40,79–81,189–192] and DOX- pORF-hTRAIL conjugate [81]	Polyamino terminated PAMAM [38]; polyamino terminated PPI with the periphery partly modified by grafting dextran [39]; PPI dendrimers with polyamino and polyfolate peripheries [40]; polyamino terminated PAMAM with the periphery partly modified by grafting the peptides HAIYPRH [80,81] or LFC131 [189], or YLFFVFER (H6) [190], or RGD [79]*via* PEG spacers [80,81,190]; assymetric polyamino terminated PAMAM, one of the dendrons of which is composed of C18 alkyl chains [191]; PAMAM with the periphery sequentially modified with fluorescein isothiocyanate (FI) and LA and polyethylene glycol (PEG)-linked LA, PEG-LA), followed by acetylation [192]	*In potus*[38–40,79–81,189–192], *in vitro* [38–40,79–81,189–192], *in vivo* [38,39,80,81,191]
	Etoposide [193]	PAMAM with poly(ε-caprolactone)-*co*-PEG periphery [193]	*In potus*[193], *in vitro*[193]
	5-FU [43–46,180,194]	Polyamino terminated PAMAM [44–46]; PAMAM with FA and FA-PEG-*N*-hydroxy-succinimide peripheries [43]; polycarboxyl and polyhydroxy terminatedPAMAM [44], PEGylated PAMAM [46]; polyamino terminated PAMAM partly modified by grafting poly(N-isopropylacrylamide) [180]; PAMAM modified by grafting both poly(2-(*N,N*-diethylamino)ethyl methacrylate) (PDEA) and MeO-PEG chains [194]	*In potus*[43,45,46,180,194], *in vitro* [43,45,46,180]*in vivo*[43,46,194], *in ex vivo*[44]
	6-Mercaptopurine [96]	Polyamino terminated dendrimers composed of melamine, piperazine and piperidine [96]	*In vivo*[96]
	MTX [16,35–37,49,96]	Polyhydroxy and poly-FA terminated PAMAM [16]; PEPE dendrimers composed of PEG spacers and peripheries, 3,5-dihydroxybenzoic acid {DHBA), 2,2-bis(hydroxyl methyl) butyric acid (BHBA) and gallic acid used as branching points and cores comprising	*In potus*[16,35–37,49], *in vitro* [16,35–37,49], *in vivo*[37,96]

(continued on next page)

Table 1 *(continued)*

Aimed therapeutic activity	Encapsulated FDA-approved AP	Type of dendrimer structure	Type of test performed[a]
		butane-1,2,3,4-tetracarboxylic (BTCA) and aspartic acids [35]; PEPE dendrimers composed of PEG spacers and glucosamine-PEG peripheries, DHBA and gallic acid used as branching points and cores comprising BTCA and aspartic acids [36]; PAMAM dendrimers with tBoc-NH-PEG periphery [37]; polyamino terminated PAMAM [49]; polyamino terminated dendrimers composed of melamine, piperazine and piperidine [96]	
	PTX [41,42,104,184]	Polyhydroxy terminated polyglycerol dendrimers [41,42]; polyhydroxy terminated dendrimers composed of triazine cycles and diethanolamine spacers [104]; polycarboxyl terminated PPI with the peryphery partly modified by monoclonal antibody mAbK1 [184]	*In potus*[41,42,104,184], *in vitro*[104,184], *in vivo*[104,184]
	Tretinoin [49]	Polyamino terminated PAMAM [49]	*In potus*[49], *in vitro*[49]
Antiretroviral	Efavirenz [195]	Polamino terminated PPI with the periphery partially modified by grafting tuftsin [195]	*In potus*[195], *in vitro*[195]
	Lamivudine [82]	PPI with polyamino and polymannosylate peripheries [82]	*In potus*[82], *in vitro*[82]
	Zidovudine [196]	PPI with sialic acid conjugated-polymannosylate periphery [196]	*In potus*[196], *in vitro*[196]
Antithrombotic	Enoxaparin [64]	Polyamino terminated PAMAM [64]	*In potus*[195], *in vitro*[195]
Antitubercular	Rifampicin [197]	Polymannosylate terminated PPI [197]	*In potus*[197], *in vitro*[197]
Antiulcer	Famotidine [114]	Polyamino terminated PPI [114]	*In potus*[114]
Diuretic	Furosemide [198]	Polyamino and polymethyl ester terminated PAMAM [198]	*In potus*[198]
Immunosuppressive	Mycophenolic acid [57]	PAMAM with polyamino and polyacetyl peripheries [57]	*In potus*[57]
Lipid-lowering	Simvastatin [199]	Polyamino terminated PAMAM [199]	*In potus*[199], *in vitro*[199], *in vivo*[199]
Myotic and mydriatic	Pilocarpine nitrate [74]	PAMAM with polyamino, polyhydroxy and polyacarboxylate peripheries [74]	*In potus*[74], *in vivo*[74]
	Tropicamide [74]	PAMAM with polyamino, polyhydroxy and polyacarboxylate peripheries [74]	*In potus*[74], *in vivo*[74]
Vitamines	β-Carotene [98]	PEPE dendrimers composed of PEG spacers and peripheries, DHBA used as branching points and cores comprising BTCA and aspartic acid residues [98]	*In potus*[98]
	Nicotinic acid [65]	Polyamino terminated PAMAM [65]	*In potus*[65]

[a] Herein, the term "*in potus*" (*potus,* lat. – drink, liquid) is chosen to designate AP-encapsulation/release tests in model systems (solutions) without resorting to the use of any complex biological object (*e.g.*, cell, tissue, organ, animal as *in vitro*, *in ex vivo* and *in vivo* experiments, respectively).

release mechanism has shown some interesting early promise with dendrimers encapsulating APs *via* electrostatic interactions [15,40,47,68,106]. Temperature [47,106] and GSH sensitive [169] release have also been proposed for antineoplastic drug release, as shown with dendrimers bearing *N,N*-dimethylaminoethyl methacrylate, thioether and disulfide groups, respectively. A more recent strategy to deliver APs to tumors using dendrimers consists in active (ligand-mediated) targeting of the pathological site. The presence of such ligands (*i.e.*, antibodies, peptides, aptamers, polysaccharides, FA, *etc.*) should increase retention and accumulation of dendrimers in the targeted tissues and allow a selective and efficient cellular uptake [15,16,39,40,43,60,80–82,154,184,189,190,192,195–197].

2.3.2.2. Encapsulating APs for other chemotherapies. Besides anticancer therapy, there are other areas in clinical medicine that might also benefit from improved pharmacological parameters of APs achieved through encapsulation in dendrimers (Table 1). For example, the most frequently reported beneficial effects are the sustained AP-release and the significant increase in aqueous solubility of the inclusion complexes containing highly hydrophobic APs, compared to the non-formulated drugs. The encapsulation can also help to better control the crossing of biological barriers for local administration (*i.e.*, *via* skin [63], lungs [64], leading to enhanced AP systemic levels, as well as *via* ocular cornea [74,105], by allowing higher quality of AP-distribution in targeted organ, respectively) that is potentially suitable for many therapeutic applications, *e.g.*, antibacterial [105], antiinflammatory [63], and antithrombotic [64], diagnostics [74], *etc.* (Table 1). The presence of larger fenestrations between epithelial cells of blood vessels into inflamed tissues (analogously to the EPR effect) favours a more selective penetration and accumulation of AP-dendrimer complexes into affected regions. This approach was proposed for the delivery of APs to arthritic regions [59,60,70,114], especially when the passive accumulation approach is combined with the possibility of the active targeting using the FA-ligand [60]. An effective conjunction of the increase in AP-solubility, prolonged AP-release and lower hemolytic activity represents the basis of testing dendrimer-based formulations in treating malaria [97,176,177]. Also, the capability of polymannosylated dendrimers to interact with HIV-infected monocytic macrophages was used to target an antiretroviral AP [82,196].

3. Main hurdles and prospects for future developments

By summarizing the results shown in previous sections, it is reasonable to conclude that dendrimers employed as non-covalent AP-encapsulation agents constitute a very promising class of materials, and that it is only a question of time before they start being better represented in the pharmaceutical market. Among the major hurdles to this achievement, including the current lack of clinical trials, there are some fundamental factors, the aspects of further development related to which will be discussed in detail below.

3.1. Aspects of chemical synthesis and purification

During the past 40 years, the synthesis of dendrimers has evolved into a mature field of chemistry. Significant progress has been made in elaborating general strategies of efficient macromolecular assembly, including both the classical 'divergent' and 'convergent' motifs, as well as the more recent 'by blocks' methods [5,69,98,200], Fig. 6. Complementary structural variations can also be achieved using the 'by sectors' and/or 'by layers' assembly approaches [5], Fig. 6. In addition, we can confidently claim that the extent of molecular diversity of

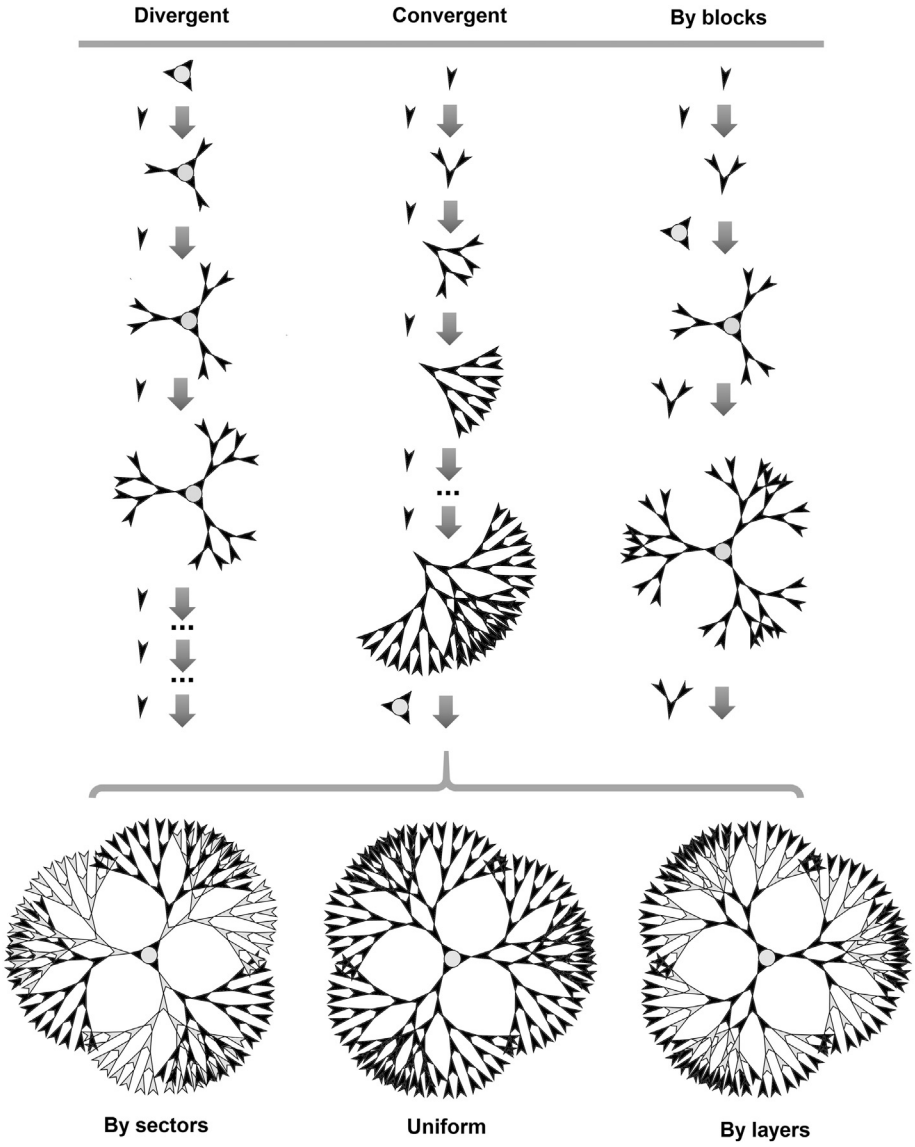

Fig. 6. General schematic representation of the chemical assembly of dendrimers from trifunctional monomer and central core units, using different methods.

dendrimers is still far from being reached [88], which is not surprising, given the consistency of new discoveries in the fields of chemical catalysis and synthesis of new heteroatom architectures.

From a practical standpoint, the choice of design strategy is governed by the nature of the structural constituents, their location in the macromolecule as well as by the G value. As a general rule, the *dense packing state theory* of De Gennes and Hervet [201] stipulates that for each type of hyperbranched structure there is a limit value of G, G_{lim}, at which the complete chemical modification of the dendrimer's surface is impossible due to steric hindrance. However, in practice, it is quite frequent to observe difficulties with obtaining pure dendrimers at $G < G_{lim}$ (*e.g.*, for PAMAM, G_{lim} is 10, while the presence of incompletely substituted homologues is usually observed at G7 [118]). Noteworthy, in real systems, the highest G values are mostly achieved *via* divergent methods, which is probably due to the fact that the monomer units forming the dendrimer scaffold are less bulky compared to the dendrons and molecular blocks employed by other synthesis approaches (Fig. 6). On the other hand, the divergent and 'by blocks' methods are characterized by higher purity of the final macromolecular structures and by a much smaller number of reaction steps [98,200]. Therefore, for a dendrimer synthesis on an industrial scale, the 'by blocks' approach could be considered as a better compromise potentially allowing reduction of labour time and cost savings.

In addition to the synthesis aspects, the efficacy of a large-scale chemical production is also determined by the possibility of employing inexpensive and reliable purification procedures. In the case of dendrimers, nearly always presented as *perfectly defined structures*, it is still, however, a very challenging task. As the generation number increases, the structural difference between desired products and their side products of incomplete transformation of the previous generation becomes increasingly negligible, which makes the isolation of pure dendrimers almost impossible using conventional methods. Given that implementation of more sophisticated supplementary purification protocols is often associated with a significant increase in production costs, the manufacturing of pseudo-dendrimers (mixtures of nearest homologues of aimed dendrimers, see also in Section 2.1.1.2) could represent a very promising and satisfactory economic alternative [107].

3.2. Aspects of AP-retention efficacy

In the context of AP-delivery, the retention efficacy determines the ability of dendrimers to accommodate a certain amount of AP and to retain it not only for storage purposes, but also until it reaches its therapeutic target after medical administration. A typical procedure of non-covalent encapsulation consists of solubilizing both dendrimer and AP in a suitable medium, and then of removing non-solubilized

ingredients. Generally, encapsulation rates and stability of resulting inclusion complexes increase with G-value of dendrimers and with their number of groups responsible for the stability of the complex. However, except for few rare cases associated primarily with the creation of strong steric hindrance [39,75], non-covalent association of dendrimers with AP is often characterized by low complex stability leading to a too-rapid and uncontrollable AP-release even in the most simple model tests. It should be noted, however, that in the overwhelming majority of the studies, the exact impact of the dendrimer structure on the AP-encapsulation process remains to be determined. The strategy of elaborating dendritic vectors based on the quantitative evaluation of structural factors, however represents a logical pathway in developing new better performance systems, is still rather rare. Nevertheless, it is reasonable to assume that potentially, such a systematic approach to solving problems with AP-retention efficacy could lead to the most effective outcomes. In particular, a possible solution would be to design macromolecules possessing the structural elements and their locations specifically optimized for capturing the target AP, thus, by using an individual approach to each payload. A significant help in this approach can be provided by methods of computational chemistry, namely, molecular mechanics techniques employing protocols of molecular dynamics with geometric optimization under variation of external conditions such as temperature and medium composition [55,102,202]. Analogously, for this purpose, some methods of theoretical chromatography addressing *structure-retention relationships* for predicting the quality of separating mixtures of analytes using different stationary and mobile phases [203] can also be potentially adapted.

Attention should also be paid to the composition of the model release media used for preliminary assessment of the AP-retention efficacy. As presented in Section 2.1.2, the more advanced approaches of AP-release modeling are currently based on considering the influence of such important external factors as the presence of electrolytes, the influence of plasma proteins, pH, and temperature. The possibility of supramolecular aggregate formation by dendrimers, which remains poorly understood, could also contribute to the AP-retention process (Section 2.2). Therefore, the ultimate objective of these preliminary tests should be to maximize as much as possible the similarity with the biological systems.

3.3. Aspects of dendrimer-mediated targeting of pathological sites

Among all the parameters used to assess the efficacy of AP-vectors, the capability of precisely targeting pathological sites (resulting respectively from the vector's bio-distribution and bio-adsobtion properties) may be considered as the most important characteristic. Nevertheless, the guaranty of selective AP-delivery to a single site by *passive* and/or *active transport* strategies, is still far from being realistic for many therapeutic treatments reported so far [31,32,204]. In this respect, the use of dendrimers does not represent an exception, by following the same general trends intrinsic to other nano- and submicron AP-vectorization platforms. Generally, their targeting capacities are limited to specificity of the organism physiology, as well as to the possibilities of employing the difference in biological parameters related to pathological changes *versus* healthy cells and tissues. As presented in Section 2.3.2, dendrimers have already demonstrated the ability to improve APs' aqueous solubility, permeability to some biological barriers as well as allowed more prolonged AP release. Preliminary tests on small animal models have demonstrated that the most efficient strategy to improve AP-bio-distribution using dendrimers is the combination of both, passive and active targeting approaches, in particular, by the fine-tuning of vector's size and surface properties (Section 2.3.2).

Regarding the immediate future of studying dendrimers as non-covalent AP-vectors in specific therapeutic treatments, it is reasonable to assume that along with already actively developing pharmacological areas (such as antineoplastic, anti-inflammatory, antimalarial, *etc.*, see Table 1), other possible medication uses will also be tested. In particular, the most likely pharmacological targets for further *in vivo* experiments could be external disorders, as well as diseases, blood, blood vessels, or/and liver pathologies, in the case of which the AP-delivery is not associated with important difficulties. For example, by taking into account some encouraging results obtained *in vitro* (Section 2.3.2), treatment of infections caused by viruses, bacteria or fungi is potentially of strong interest. In addition, dendrimers themselves are found to be effective against bacterial cultures, when their periphery is polycationic [205], as well as potential anti-inflammatory [206] and anti-*Herpes-simplex*-virus [207] agents when presenting anionic (sulfonic acid) surface groups (see also about the commercial topical antiviral Vivagel™ and an experimental anticancer PHSCN-lysine dendrimer in Section 2.3.2). Also, dendrimers, as stable nano-sized particles, could be suitable candidates for penetrating through viscous biological media such as mucus, which could be helpful in eliminating pathogenic biofilms [208], as well as in treating disorders characterized by formation of thick mucus (*i.e.*, in lungs, as observed, *e.g.*, in cystic fibrosis (*CF*) patients). In addition to the potential AP-vectorization functions (*e.g.*, in delivering cystamine [209]) possible concomitant beneficial effect of using dendrimers in treating *CF* symptoms could be the fact that polycationic macromolecules (including dendrimers) exert a strong compaction effect on deployed polyanionic DNAs (the main ingredient responsible for the high viscosity of *CF* lung sputum [210]), thereby facilitating their effective removal from aqueous solutions [211]. Another potentially interesting trend in targeting pathological sites is the use of external remote guidance, *i.e.*, by localized magnetic field, as demonstrated recently with some AP-charged nanoparticles and bacteria [212]. In the case of dendrimers, however, this area is still very poorly studied, despite solid advances in creating magnetic structures [213,214]. In the long term, one can also expect the emergence of new hybrid combinations of external guiding with passive/active AP-targeting strategies.

3.4. Aspects of controlled AP-release in vivo

Generally speaking, the aspects of controlled AP-release *in vivo* by a vector is related to its features the functions of which are diametrically opposite to those determining the AP-retention efficacy discussed above (Section 3.2). The main point is that AP-retention by the carrier should cease only upon reaching the therapeutic target, but not before then. Consequently, if effective AP release is sought, strong AP retention capacity will require even stronger changes in the vector structure, and accordingly, a stronger perturbation factor triggering the release. For simplicity, controlled AP release processes can be divided into two main groups: the ones triggered by internal factors, which are already present into the host organism, and the ones requiring a supplementary external input. Internal triggers comprise the use of physiological agents either influencing only the stability of dendrimer-AP-inclusion complexes, with the preservation of the general chemical integrity (*i.e.*, pH, the presence of compounds having more affinity to the dendrimer than the encapsulated APs, *etc.*), or leading to its degradation (*i.e.*, achieved by the sensitivity of some dendrimers to carboxyesterases and GSH), as shown in Sections 2.1.2 and 2.3.1.2. It is worth noting, however, besides the efforts to design systems sensitive to the internal parameters mentioned above, differences in bio-distribution compared to controls is usually not significant [31,32,204]. Therefore, for a very localized AP release, it is preferred to provide the vector with additional features, such the ones responsible for an adequate targeting of pathological sites, as discussed in Sections 2.3.2 and 3.3. The external triggering can be achieved by localized ultrasounds, temperature, UV-radiation, alternating magnetic or electric fields, as already tested on different AP-delivery platforms, including dendrimer-based ones [127,128,215,216]. Despite complementary costs and eventual technical problems, this approach is potentially very promising, given the possibility of accurately targeting areas of action. In this context, it is

also reasonable to expect the emergence of systems based on dendrimers sensitive to the external stimuli, as well as the appearance of new structures exploiting combinations of both internal and external triggering approaches, *e.g.*, for a controlled release of different APs from the same vector.

3.5. Toxicological aspects

As already mentioned in Section 2.3.1.2, there is currently a lack of clear enough information to draw conclusions yet on the overall impact of dendrimers as AP-vectors on human health, especially over the long term. At the same time, toxicological issues represent a very serious factor to hinder their transfer from the laboratory bench to the bedside. The data obtained using *in vitro* and *in vivo* models suggest that the surface properties, generation, concentration, and duration of exposure, as well as the bio-degradability of internal chemical bonds and the biological properties of the degradation products, are the key parameters determining the general toxicological profile of these macromolecules. Most comparative studies showed that the polycationic peripheries of dendrimers are worse short-term tolerated than neutral or acidic ones. As for long-term effects, the most promising are macromolecules degradable over time under biologically relevant conditions, allowing the formation of harmless low molecular weight products, capable of being either easily eliminated directly by renal clearance, or reused usefully by the organism for its own biological needs (*e.g.*, as proposed in the case some polyester scaffolds, Sections 2.1.1.2 and 2.3.1.2). In addition, it is worth mentioning that these general rules were not always confirmed by experimental results. A few toxicological studies *in vitro*[111,142,143] and *in vivo*[142] performed with polyester dendrimers and pseudo-dendrimers possessing neutral hydrophilic surfaces showed serious side effects, which are not usually characteristic of this type of structure. Reproducibility problems of cytotoxicity tests involving dendrimers, depending on the cell-line, were raised first by R. Duncan and L. Izzo [129]. These problems involve the mechanisms of endocytosis occurring during the internalization of macromolecules which could be different from one cell line to another. Towards the toxicological aspect of elaborating dendrimers as AP-delivery systems, it is also important to take into account that the total absence of any adverse action (if possible at all), although very desirable, should not be considered as essentially mandatory. Given the other decisive aspects discussed above, it becomes relevant rather to minimize possible biological risks, by finding an optimal compromise between the impact of dendrimer itself on the organism and its performance as an effective AP-vector upon the whole.

4. Conclusions

The significant amount of information amassed to date indicates that dendrimers are potentially of great interest as promising excipients for the non-covalent encapsulation and targeted delivery of APs. Despite their rather complex architecture, these nano-sized fractal-like macromolecules are characterized by a very high-level of control and regularity, allowing the fine-tuning of structure, including the insertion of desired molecular segments and chemical bonds between them, offering a fine control over parameters of internal scaffold architecture and periphery, which finally determine their ability to act as AP-vectors. Compared to other AP-delivery platforms, especially multimolecular ones (*e.g.*, liposomes and micelles), dendrimers possess higher stability *in vivo* and could be employed to reach high reproducibility in results, without resorting to intricate and costly procedures for an eventual AP-formulation on an industrial scale. As non-covalent AP-encapsulating agents, dendrimers already demonstrated the abilities to increase the solubility of highly hydrophobic APs in aqueous media, to reduce the plasma peak concentration related to systemic cytotoxicity, as well as to improve AP-bio-distribution *in vivo*. Despite the successes achieved, the translation of dendrimers to the market is still hindered by many factors, such as the need of multi-step chemical synthesis and purification processing, insufficient AP-retention efficacy *in vivo* and poor targeting of pathological sites (the last one remains intrinsic to most known AP-encapsulation platforms). In this context, it should however be noted that the global multifaceted potential of dendrimers is still far from being fully explored in many aspects, including the possibility of solving the most commonly cited problems. Thus, our attempt to combine in the present review the main prerequisites and prospects for further development of dendrimers as non-covalent AP-delivery agents allows us to conclude that in the near future, it is reasonable to expect from them new very strong and promising results.

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