ABSTRACT

Dendrimers are synthetic macromolecules composed of repetitive layers of branching units that emerge from a central core. They are characterized by a tunable size and precise number of peripheral groups which determine their physicochemical properties and function. Their high multivalency, functional surface and globular architecture with diameters in the nanometer scale makes them ideal candidates for a wide range of applications. GATG (gallic acid-triethylene glycol) dendrimers have attracted our attention as a promising platform in the biomedical field because of their high tunability and versatility. The presence of terminal azides in GATG dendrimers and PEG-dendritic block copolymers allows their efficient functionalization with a variety of ligands of biomedical relevance including, anionic and cationic groups, carbohydrates, peptides or imaging agents. The resulting functionalized dendrimers have found application in drug and gene delivery, as antiviral agents and for the treatment of neurodegenerative diseases, in diagnosis and as tools to study multivalent carbohydrate recognition and dendrimer dynamics. Herein we present an account on the preparation and recent applications of GATG dendrimers in these fields.

KEY WORDS: dendrimer, block copolymer, drug delivery, multivalency, NMR

ABBREVIATIONS: AMCA, Aminomethylcoumarin; CA, HIV capsid protein; CTD, C-Terminal domain; Con A, Concanavalin A; CuAAC, Cu(I)-catalyzed azide-alkyne cycloaddition; DTPA, Diethylenetriaminepentacetate; DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; DO3A, Tri-tert-butyl 1,4,7,10-tetraazacyclododecane-1,4,7-triacetate; EGFP, Enhanced green fluorescent protein; FDA, Food and drug administration; FITC, Fluorescein isothiocyanate; GATG, Gallic acid-triethylene glycol; G_n, Dendrimer generation, n denotes the generation number; Glc, Glucose; HEK293T, Human embryonic kidney cell line 293T; HIV, Human immunodeficiency virus; HPMA, *N*-(2-hydroxypropyl)methacrylamide; HSV, Herpes simplex virus; ITC, Isothermal titration calorimetry; Man, Mannose; Mor, Morpholine; MRI, Magnetic resonance imaging; NOE, Nuclear Overhauser effect; PAMAM, Polyamidoamine; PEG, Poly(ethylene glycol); PPI, Polypropyleneimine; PIC, Polyion complex; RGD, Arginylglycylaspartic acid; SPR, Surface plasmon resonance.

INTRODUCTION

Dendrimers are synthetic tree-like macromolecules composed of repetitive layers of branching units that emerge from a central core (Fig. 1). They are synthesized in a controlled iterative fashion through generations with nil dispersity, precise molecular weight, and discrete properties (1-3). Their high functional surface, globular architecture in the nanometer scale, and inherent multivalency makes them ideal candidates for a wide range of applications, from bio- and nanotechnology (4-8) to catalysis and materials science (9-11). The first reports on

dendrimers were published independently in the late 70s and early 80s of the last century by the groups of Vögtle (12), Newkome (13), and Tomalia (14). Since then, over a hundred dendritic architectures have been described in the search of improved and novel properties. Among them, recognized dendritic families include polyamidoamine (PAMAM) (15), polypropyleneimine (PPI) (16), and others based on polyamide (13), polyether (17), polyester (18, 19), and phosphorous-based (20) scaffolds.

Recently, we have turned our attention to the GATG (gallic acid-triethylene glycol) dendritic family as a promising platform in the biomedical field (Fig. 1). GATG dendrimers, first described by the group of Roy (21-23), are composed of a repeating unit carrying a gallic acid core and hydrophilic triethylene glycol arms with terminal azide groups. Advantage of the azides is taken for the dendritic generation growth, easily accomplished by a reduction/amide coupling sequence, as well as for the dendrimer decoration by means of the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (24-28) as demonstrated by our group. As part of our effort to develop the biomedical applications of GATG, we have also described the incorporation of the FDA-approved poly(ethylene glycol) (PEG) at the focal point of dendritic wedges to render PEG-GATG block copolymers with increased solubility and stealth properties. PEG-dendritic block copolymers constitute interesting hybrid structures where differences in the solubility properties of the blocks can be exploited in the preparation of micelles and other nanostructures of biomedical interest (29-31).

Herein we present an account of our journey with GATG that focuses on recent examples in the fields of drug and gene delivery, diagnosis, and antiviral activity, as well as the use of GATG as tools to study biological processes and the dynamics of dendrimers.

SYNTHESIS OF GATG DENDRIMERS AND PEGYLATED BLOCK COPOLYMERS

GATG dendrimers ([Gn]-N_3, where n is the generation number) are synthesized divergently from a repeating unit shown in Fig. 1, following a straightforward azide reduction/amide coupling sequence. Initial reports by the group of Roy in the 90's described GATG sialodendrimers and dendronized chitosans up to the second generation (G2) as promising microbicides (21-23). Nevertheless, the synthesis of the repeating unit (four steps from triethylene glycol, 23% overall yield) proved to be a hurdle in accessing large quantities and higher G of these dendrimers, which finally hampered their subsequent development.

Aware of these limitations, in 2006 our research group described an improved preparation of this repeating unit from commercially available chlorotriethylene glycol in 77% overall yield (32). By observing green chemistry principles (atom economy, safety, waste reduction), this synthetic route has been further developed for the cost-effective production of the repeating unit in batches larger than 100 g in excellent overall yield (86%) and purity (Fig. 1) (33). With an easy and scalable access to the repeating unit, the preparation of GATG dendrimers and PEG-

dendritic block copolymers has been efficiently achieved in large quantities up to G4 (243 peripheral azides) (32, 34, 35). The PEGylated block copolymers are synthesized following a "chain first" approach where the PEG is initially incorporated at the focal point of a GATG repeating unit and then, the higher G obtained divergently. Noteworthy, PEG facilitates the purification steps in the synthesis of the block copolymers thanks to its properties as a soluble polymeric support (36). As mentioned, the surface functionalization of GATG dendrimers and block copolymers by CuAAC with unprotected ligands (carbohydrates, anionic and cationic moieties, peptides, imaging agents) has proceeded straightforward in our hands, allowing the preparation of a variety of functional dendritic structures of biomedical interest that will be presented in the following sections.

CELLULAR INTERNALIZATION OF GATG DENDRIMERS

Dendrimers have traditionally found great attention in targeted drug delivery, particularly for cancer therapy and diagnosis (5, 6). The high number of functional groups on their periphery allows the simultaneous incorporation of bioactive molecules, cytostatic drugs, diagnostic probes and targeting ligands. In addition, the combination of high water solubility and hydrophobic cores is well suited for the covalent attachment or physical encapsulation of large payloads of therapeutic molecules. Cellular uptake of dendrimer-based drug delivery systems has proved significantly higher than that of linear polymeric carriers (37, 38), which can be explained on the basis of their compact nano-sized architecture in solution.

In order to explore the versatility of PEG-GATG block copolymers as tools in the biomedical field, we have analyzed in collaboration with Albertazzi their cellular internalization and intracellular fate (39), both properties of particular interest in drug delivery. To this aim, a PEGylated block copolymer of third generation (PEG-[G3]-N_3) was functionalized *via* CuAAC with different peripheral groups, including neutral and charged moieties, biologically active carbohydrates, and peptides. All dendritic structures were labeled with fluorescein (FITC), and the effect of the surface functionalization on cell-uptake and intracellular trafficking was studied by confocal microscopy in HeLa cells. It was observed that, while cationic PEG-[G3]-NH_3^+ showed a strong internalization consistent with its ability to bind cell membranes through ionic interactions, anionic PEG-[G3]-OSO_3^- displayed only a weak internalization because of its lower affinity for cell membranes. This behavior was even more evident for a neutral acetylated PEG-[G3]-NHAc that exhibited no internalization at all. In addition, the intracellular final fate of PEG-[G3]-NH_3^+ was tracked, revealing colocalization with lysosomes. The effect of the surface functionalization with biologically relevant ligands, such as lactose and a cyclic RGD peptide was also investigated (39). Confocal microscopy and flow cytometry assays showed significant cellular uptake in HepG2 cells for dendritic structures decorated with lactose and in HeLa cells for those containing the RGD sequence. Based on these results, a prototype of drug

carrier capable of selectively entering cells and specifically releasing its payload in the acidic lysosomal environment was designed from PEG-[G3]-NH_3^+. With this aim, a coumarin dye was bound through a pH-sensitive hydrazone linker to the dendritic platform (Fig. 2). The performance of this system was evaluated with a double fluorescent-labeling allowing for simultaneous monitoring the localization of the dendritic carrier (FITC) and the coumarin dye as a model cargo molecule (AMCA-hydrazide). Fluorescence experiments at short incubation times showed high colocalization between FITC and AMCA in the endolysosomal system. However, reduced colocalization and increased fluorescence intensity due to AMCA could be observed at longer incubation times (Fig. 2), which are consistent with a lysosomal hydrolysis of the hydrazone linkers and subsequent release of AMCA to the cytoplasm. This GATG-based conjugate accomplishes the main requirements for a successful drug delivery system (cell internalization, intracellular release, endosomal escape) and represents a promising proof-of-principle for further applications of GATG dendrimers and block copolymers in drug delivery.

GATG NANOSTRUCTURES FOR DRUG AND GENE DELIVERY

PEG-GATG block copolymers are especially suited for drug and gene delivery applications. For instance, they have been used in the preparation of polyion complex (PIC) micelles. PIC micelles are smart delivery systems originally described by the groups of Kataoka and Kabanov (40, 41), that are formed by electrostatic interaction between oppositely charged polyions. Similarly to classical polymeric micelles, PIC micelles have a core-shell structure with a core of ionic blocks surrounded by a neutral hydrophilic corona, typically of PEG. Properties such as their small size, electrical neutrality, and narrow size distribution make these systems highly attractive for drug delivery applications (42, 43).

Our research group has reported the preparation of nanosized PIC micelles from an anionic PEG-GATG block copolymer of G3 decorated with 27 peripheral sulfates and poly-*L*-lysine (PLL) as a model polymer of opposite charge (Fig. 3) (44). Notably, these micelles displayed enhanced stability towards ionic strength compared to conventional PIC micelles from linear copolymers, a fact has been ascribed to the more rigid dendritic architecture. These micelles are envisioned as attractive delivery systems for low molecular weight drugs, proteins, nucleic acids and imaging agents. In another example, making use of the developed CuAAC conditions for the anionic decoration of GATG dendrimers, carboxylates have been introduced onto the dendritic periphery of PEG-GATG copolymers, which has allowed the preparation of related pH-sensitive PIC micelles with potential applications in cancer therapy (45).

Cationic synthetic carriers have been widely assayed in the last decade as an alternative to viral vectors for the delivery of nucleic acids (46). Among them, cationic dendrimers with the ability to electrostatically interact with negatively charged nucleic acids have received special attention (7, 47). The complexes, named dendriplexes, obtained from commercially available

PAMAM and PPI dendrimers have been by far the most investigated ones. Unfortunately, some limitations have arisen, mainly associated to the excess of positive charge necessary to efficiently complex the genetic material, which results in aggregation with blood components and cytotoxicity. One strategy to overcome these limitations has been the use of PEGylated cationic block copolymers to mask the positive charge (48, 49). Similarly to PIC micelles, the positively charged dendritic block interacts with the negatively charged nucleic acid forming an inner core surrounded by a hydrophilic PEG corona. The obtained dendriplexes are sterically stabilized and present lower z-potential, reduced cytotoxicity, and increased circulation times.

In light of these results, cationic GATG dendrimers and their block copolymers appeared to be excellent candidates for gene delivery applications. Amine-decorated GATG scaffolds are easily obtained by reduction of the terminal azides. This results in a high positive charge in physiological media and so, in the ability to condense and protect nucleic acids. In addition, the hydrophobic nature of the gallic acid was envisioned to enhance the cellular uptake and transfection efficiency of the dendriplexes. In collaboration with the group of Alonso, we have recently evaluated the ability of amino-functionalized GATG dendrimers and block copolymers to complex plasmid DNA (pDNA) (34, 50). As a result of an analysis of the influence of G, N/P ratio, and the presence/absence of PEG on the dendriplex size and z-potential, we have proposed these dendriplexes as core–shell nanostructures with sterically induced stoichiometry. A single pDNA condensed at the core surrounded by a shell of dendrimers with a stoichiometry determined by the core/dendrimer relative size: the higher the dendrimer G, the fewer the dendrimers that can be accommodated on the dendriplex surface (Fig. 4). Interestingly, in the case of PEG-dendritic block copolymers, this implies the possibility of tuning the PEG density on the dendriplex surface, which may be of interest to control the stealth properties for specific gene therapy applications.

The stability, cytotoxicity and interaction with blood components of the dendriplexes were studied, along with their ability to transfect mammalian cells (50). It was revealed that the dendriplexes formed from GATG dendrimers are stable, biocompatible and do protect pDNA from degradation. More importantly, dendriplexes were effectively internalized by HEK-293T cells, which were successfully transfected. It was also observed that PEGylation remarkably influences the properties of the dendriplexes. As previously seen in other nanostructures, PEG improves the biocompatibility at the cost of a reduced cellular uptake (51). Our results highlighted that the PEGylation degree of nanostructures should be carefully adjusted in order to obtain an optimized stealth formulation without compromising the transfection efficiency. A straightforward approach for modulating the density of the PEG shell that ensured a successful transfection was developed by employing mixtures of GATG dendrimers and PEGylated copolymers for the preparation of the dendriplexes (Fig. 4). Further investigations, including *in*

vivo studies, are planned to draw definite conclusions on the efficacy of this mixed stealth formulation in animal models.

GATG DENDRIMERS AS DRUGS: INTERACTIONS WITH PROTEINS AND PEPTIDES

Besides the aforementioned application of dendrimers as drug carriers, they can also act as drugs by themselves (8). For instance, several dendritic structures have shown promising antimicrobial and antibacterial activity that pave their way as alternatives to conventional antibiotics (52-54). Anionic dendrimers with anti-inflammatory properties (55, 56) or as agents for the multiplication of human natural killer cells (57) have been also described. The use of dendrimers as inhibitors of viral infections has been investigated as well, in particular against the human immunodeficiency (HIV) and herpes simplex viruses (HSV-1 and HSV-2) (58-60). VivaGel^{TM}, a L-lysine-based dendritic microbicide decorated with anionic groups, undoubtedly constitutes the most relevant example. It has been evaluated in Phase II clinical trials as a vaginal gel for preventing/reducing transmission of HIV and genital herpes (61). There are also reports on the use of dendrimers that dissolve prion-protein aggregates and hamper fibril formation of prion and β-amyloid (Aβ) peptides (62, 63).

In this context, our group together with those of Velázquez-Campoy and Neira were encouraged to explore the ability of GATG dendrimers to interact with HIV-1. We hypothesized that if dendrimers can destabilize the tertiary structure of proteins, they could also disrupt the quaternary structure of the capsid protein CA of HIV-1 and hamper its assembly to form the viral capsid. Thus, CA has recently emerged as a promising target for the development of new anti-HIV drugs based on its critical role during HIV morphogenesis (64). Our results demonstrate that G1 GATG dendrimers bind to the C-terminal domain of CA (CTD) with a dissociation constant in the micromolar range, as shown by isothermal titration calorimetry (ITC) (65). The affinity of some of the dendrimers for CTD was similar to that of synthetic peptides binding the dimerization region, and of comparable magnitude to the homodimerization affinity of both CTD and CA. More importantly, a G1 dendrimer decorated by CuAAC with peripheral benzoate groups ([G1]-CO_2Na) was able to hamper the assembly of the HIV capsid *in vitro* (Fig. 5), in what represents the first example of a dendrimer as a lead compound for the development of anti-HIV drugs targeting the capsid assembly.

Nanomedicine has shown great potential for the treatment of many central nervous disorders, such as brain cancer, epilepsy, Alzheimer´s or Parkinson´s diseases. Among the different nanostructures employed for this purpose, dendrimers have been intensively investigated in neurodegenerative processes, especially Alzheimer's disease (66). According to the amyloid cascade hypothesis, amyloid peptide aggregation is closely related to the onset and development of Alzheimer's disease. Since Aβ peptide oligomers intermediate in the assembly of fibrils are more neurotoxic than the end products, novel strategies aimed to reduce their toxic effects by

affecting the aggregation process are of much relevance (62, 67, 68). Encouraged by the promising properties of GATG dendrimers as inhibitors of the dimerization of CA, we envisaged GATG interfering in the formation of amyloid fibrils. In a joint effort with the group of Klajnert, a morpholine-decorated GATG dendrimer ([G3]-Mor) was identified to effectively accelerate the formation of amyloid fibrils from a Aβ 1-28 peptide (thioflavin T assay, CD, transmission electron microscopy) (Fig. 6) (69). Interestingly, when the cytotoxicity of the Aβ and the pair Aβ/[G3]-Mor was monitored at different stages of the aggregation process, it was observed that [G3]-Mor significantly reduced the toxicity of the peptide, most likely by speeding up the fibril formation and lowering the concentration of the toxic prefibrillar forms in the system.

GATG GLYCODENDRIMERS AS TOOLS TO STUDY MULTIVALENT CARBOHYDRATE RECOGNITION

Carbohydrates cover a large spectrum of bioactivities from energy source and structural roles to others crucial for the development, growth, function or survival of organisms. From bacteria to mammals, cells are coated with sugars as first points of contact with their environment. Thus, they are in a position to modulate a plethora of biological processes including, cell−cell recognition, fertilization, pathogen invasion, and toxin and hormone mediation. Moreover, strong evidence suggests that carbohydrates are key diagnostic and prognostic indicators as well as therapeutic targets (70). In addition, the clustered arrangement of carbohydrates on the cell surface enables their multivalent interaction in global processes characterized by affinities and specificities much higher than monovalent interactions (71, 72). This fact has prompted the development of synthetic multivalent glycoconjugates (linear polymers, micelles, nanoparticles, nanotubes, dendrimers) with the ability to promote/inhibit biological events (73). Since carbohydrate recognition is commonly mediated by proteins (lectins), the development of more efficient diagnostic and therapeutic tools relies on a better understanding of the carbohydrate−lectin interaction. However, the complexity of the binding mechanisms associated with multivalency (intermolecular crosslinking, chelation, statistical rebinding) makes them very difficult to measure experimentally. As a consequence, binding data are frequently extracted from indirect competitive methods in solution where only relative affinities are obtained (73). In addition, these experimental designs usually represent rough models for mimicking surface-based interactions as underestimate the multivalency derived from the lectin clustering.

With the aim of gaining insight into the fundamental mechanisms of multivalent carbohydrate recognition we have synthesized GATG glycodendrimers by CuAAC from unprotected alkynated saccharides (Fig. 7) (32, 35). These were foreseen as nanotools of precise size and multivalency for mechanistic studies by surface plasmon resonance (SPR). We have

compared the outcome of carbohydrate-lectin binding studies in solution (*via* competitive experiments) and surface-bound direct experiments (with immobilized lectins on a chip surface) (74, 75). To that end, we selected the lectin Concanavalin A (Con A) and four generations of glycodendrimers carrying 3-81 mannose/glucose residues ([Gn]-Man and [Gn]-Glc). Solution experiments demonstrated the importance of multivalency in carbohydrate recognition, with affinity increases being observed from the monosaccharide to G2. The lack of further affinity enhancements at higher G, however, contrasts with the surface-bound experiments. This different outcome not only stresses the relevance of the experimental design for soluble *vs* surface-bound lectins, but also that higher dendrimer G not necessarily result in higher affinities in solution.

In the surface-bound experiments, a complex binding profile was disclosed with two limiting binding modes, a low-affinity mode associated with dendrimers binding the lectin surface monovalently, and a high-affinity mode associated with dendrimers with higher functional valency. SPR studies also revealed the dynamic nature of the binding mechanisms, with contributions depending on the glycoconjugate multivalency and lectin cluster density, but also on the local concentration of glycoconjugates in the proximity of the lectin cluster, which is a time-dependent factor (Fig. 7). As a result, an original SPR protocol was designed to gather kinetic and thermodynamic information on the interaction by analyzing the early association and late dissociation phases of the sensorgrams, areas where the low analyte concentration nearby the receptor surface favors the highest affinity binding modes. In addition, it was concluded that for surface-bound experiments, the density of receptors should be carefully selected to mimic as much as possible the biological environment if relevant quantitative information is desired beyond a list of relative affinities.

GATG AS CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) uses a strong magnetic field and radio frequency pulses to obtain internal images of organs and lesions. Differences in contrast of the images reflect the rate at which excited protons of water molecules return to the equilibrium state (relaxation times, *T*) (76). Although it is possible to obtain high quality images by manipulation of pulse sequences, high contrast is better achieved by adding exogenous contrast agents that, upon coordination to water, accelerate relaxation. Contrast agents in the clinic are based on paramagnetic ions such as gadolinium (Gd^{3+}) complexed to low molecular weight ligands (*e.g.*; DTPA, DOTA, DO3A) (77). These complexes present high relaxivity and adequate biocompatibility, stability and solubility. However, because of their small size they suffer from rapid excretion (high doses necessary) and passive distribution into the interstitial space. The use of macromolecular contrast agents is envisioned to provide longer circulation times in the bloodstream (adquisition windows) and selective diffusion through angiogenic tissue, along

297 with an increased relaxivity per Gd (78). Among the macromolecular contrast agents,
298 dendrimers are especially appealing because of their monodisperse nature and absolute control
299 of their size. Their branched structure imparts rigidity and a high density of functional groups
300 for the multivalent display of Gd and other synergistically integrated agents for therapy and
301 diagnosis. In addition, the pharmacokinetics and pharmacodynamics of dendrimers, their
302 permeability, excretion routes, and recognition by the reticulo-endothelial system can be
303 controlled by generation (78-80). The synthesis of dendritic contrast agents has been
304 conventionally achieved in a stepwise fashion through postlabeling approaches, which involve
305 the incorporation of suitable ligands onto the macromolecular scaffold, followed by
306 complexation to the desired metal ion. Unfortunately, these strategies suffer from incomplete
307 functionalization that results in mixtures of polydisperse compounds and reduced relaxivity. To
308 solve this inconvenience we have turned our attention to a prelabeling approach using Gd
309 complexes and CuAAC as an efficient coupling technology. This way, the complete
310 functionalization of three generations of PEG-GATG block copolymers was demonstrated with
311 an alkynated Gd-DO3A complex (Fig. 8) (81). The resulting monodisperse macromolecular
312 contrast agents, incorporating up to 27 Gd ions at the periphery, display molecular relaxivities
313 that increase with G up to values in the range of Gadomer-17 (82), a polylysine-based dendritic
314 contrast agents bearing 24 DO3A-Gd chelates and considered as a reference in the field. The
315 analysis of the pharmacokinetic properties of this new family of PEG-dendritic contrast agents
316 was studied in mice using a C6 glioma model (Fig. 8). After intravenous injection of the
317 contrast agents, T_1-weighted images showed similar increments of signal intensity and kinetic
318 profiles to Gadomer-17 (with maximum intensities 4 min after injection), which reveals them as
319 a promising platform for the development of dendritic contrast agents for MRI. The
320 experimental simplicity of this CuAAC-based prelabeling approach should be of relevance for
321 the preparation of alternative macromolecular metal complexes for applications in chemical
322 exchange saturation transfer MRI, fluorescence imaging, and radiolabeling.
323
324 DENDRIMER DYNAMICS
325 The flexibility of the chemical bonds within dendrimers determines their internal dynamics,
326 hydrodynamic size and topological localization of the external groups, all of relevance for
327 pharmacological properties such as biodistribution and surface accessibility. In spite of initial
328 controversies about the dendritic conformation, shape and packing, a consensus has recently
329 emerged on dendrimers as flexible macromolecular structures with a dense core and fluctuating
330 repeating unit groups (83).
331 Nuclear magnetic resonance (NMR) is a powerful tool to study the dynamics of
332 macromolecules at atomic level (84). Information is usually extracted by measuring longitudinal
333 (T_1) and transverse (T_2) relaxation times and nuclear Overhauser effect (NOE). It is especially

suited for the analysis of dendrimers since their repetitive nature offers the opportunity to probe different layers and G. Because of our interest in developing the bioapplications of GATG dendrimers, we decided to analyze their dynamical properties. With this aim we initially performed a ^{1}H NMR relaxation study in $CDCl_3$ (35). Increasing T_1 and T_2 values were observed on going from the core to the periphery that, according to the theoretical variation of relaxation times with the correlation time (τ) (Fig. 9) (85), were interpreted as a radial increase of dynamics in the same direction (congested core protons surrounded by more flexible external nuclei). This dynamical picture was later confirmed under more relevant aqueous conditions by a quantitative ^{13}C relaxation study in collaboration with the group of Widmalm (86).

In general, for NMR studies on the dynamics of macromolecules, quantitative modeling from ^{13}C relaxation is preferred to ^{1}H. However, lengthy ^{13}C experiments and the necessity of recording various parameters at different magnetic fields have limited such approach in dendrimers to a single report apart from ours (86, 87). Conversely, a great deal of information has been extracted by qualitative interpretation of ^{1}H and/or ^{13}C relaxation. These studies have, nevertheless, afforded conflicting results on the relative dynamics between the dendritic core and the periphery. With the aim of throwing light on this controversy we have recently performed a comprehensive relaxation study (^{1}H, ^{13}C; various magnetic fields and temperatures) of Fréchet-type poly(aryl ether) dendrimers (Fig. 9) (17), as an example of a dendritic family where conflicting relative dynamics between core and periphery have been reported by ^{1}H T_1 relaxation (88, 89) and by alternative techniques (90, 91). As a result of this work (92) it was revealed that NMR relaxation in dendrimers has been often misinterpreted in terms of dynamics. Dendrimers show slower dynamics at internal layers and display internal nuclei with T_2 values shorter than the periphery, but T_1 values that can be either shorter or larger depending on their position in the fast or slow motional regimes (Fig. 9). Accordingly, only the recording of T_1 data at various temperatures (alternatively, T_2 or NOE at one temperature) can ensure the correct interpretation of dendrimer dynamics. The large number of dendritic families, other than poly(aryl ether), where dynamics have been evaluated on the basis of T_1 data at one temperature urges necessity of revisiting previous NMR relaxation studies.

The fact that dendrimers obey a dense core model with increasing T_2 values from the core to the periphery has been more recently exploited in our laboratory in T_2-edited NMR experiments (93) for the stepwise filtering of the internal nuclei (94). The resulting filtered spectra benefit from reduced signal overlapping, which facilitates NMR assignment and characterization (Fig. 10). This filtering strategy has been applied to various dendritic families, nuclei (^{1}H, ^{13}C, ^{31}P) and 2D experiments (COSY and HSQC), and is envisaged to aid structural characterization and end-group analysis in related dendritic structures, including block, dendronized, and hyperbranched polymers functionalized with drugs, active targeting moieties and other labels.

CONCLUSIONS

GATG dendrimers and their PEGylated block copolymers represent promising macromolecular scaffolds for a plethora of biomedical applications. Key features in GATG are a high tunability and versatility. The presence of terminal azides in GATG allows their efficient functionalization with a variety of ligands of biomedical relevance. Depending on the desired application, GATG dendritic scaffolds have been easily decorated in a single step with anionic or cationic groups, carbohydrates, peptides or imaging agents. The resulting functionalized dendrimers have found application as nanotools to study multivalent interactions, as building blocks for the preparation of polymeric micelles and dendriplexes for gene delivery, as antiviral drugs or agents for the treatment of neurodegenerative diseases, and as contrast agents for MRI. In addition, the analysis of GATG dynamics by NMR relaxation has prompted a fundamental study on dendrimer dynamics. As a result, profound differences between the relaxation behavior of dendrimers and linear polymers were revealed that have been exploited in the filtering of NMR spectra to facilitate signal assignment and characterization.

As a concluding remark, we would like to highlight that despite the promising results of GATG dendrimers in the biomedical field, there are still ahead of us fascinating puzzles to be solved where GATG might play a role. Examples of current challenges faced by this dendritic family in our laboratory are the development of nanosystems able to tackle unresolved problems in drug delivery and to avoid the limitations derived from the potential Cu contamination after CuAAC. Regarding the first objective, advantage can be taken of the modularity of GATG dendrimers. Indeed, by making discrete structural changes, such as varying the length of the PEG chain, the dendritic generation or the hydrophobicity at the periphery, the solubility and dynamical properties of GATG can be effectively tuned. As for the second challenge, we are currently engaged in the development of Cu-free approaches that circumventing the generation of reactive oxygen species, also avoid the use of large linkers like strained cycloalkynes. Finally, we are motivated to transfer GATG dendrimers to *in vivo* situations where parameters such as, biodegradability, immunogenicity or bioaccumulation, among others, will have to be carefully evaluated to validate their clinical potential.

ACKNOWLEDGMENTS

The authors wish to acknowledge past and present lab members who have contributed to the development of dendrimers in our group. This work was financially supported by the Spanish Government (CTQ2009-10963, CTQ2012-34790, CTQ2009-14146-C02-02, CTQ2012-33436) and the Xunta de Galicia (10CSA209021PR and CN2011/037).

Conflict of Interest The authors declare that they have no competing interests.

LEGEND TO FIGURES

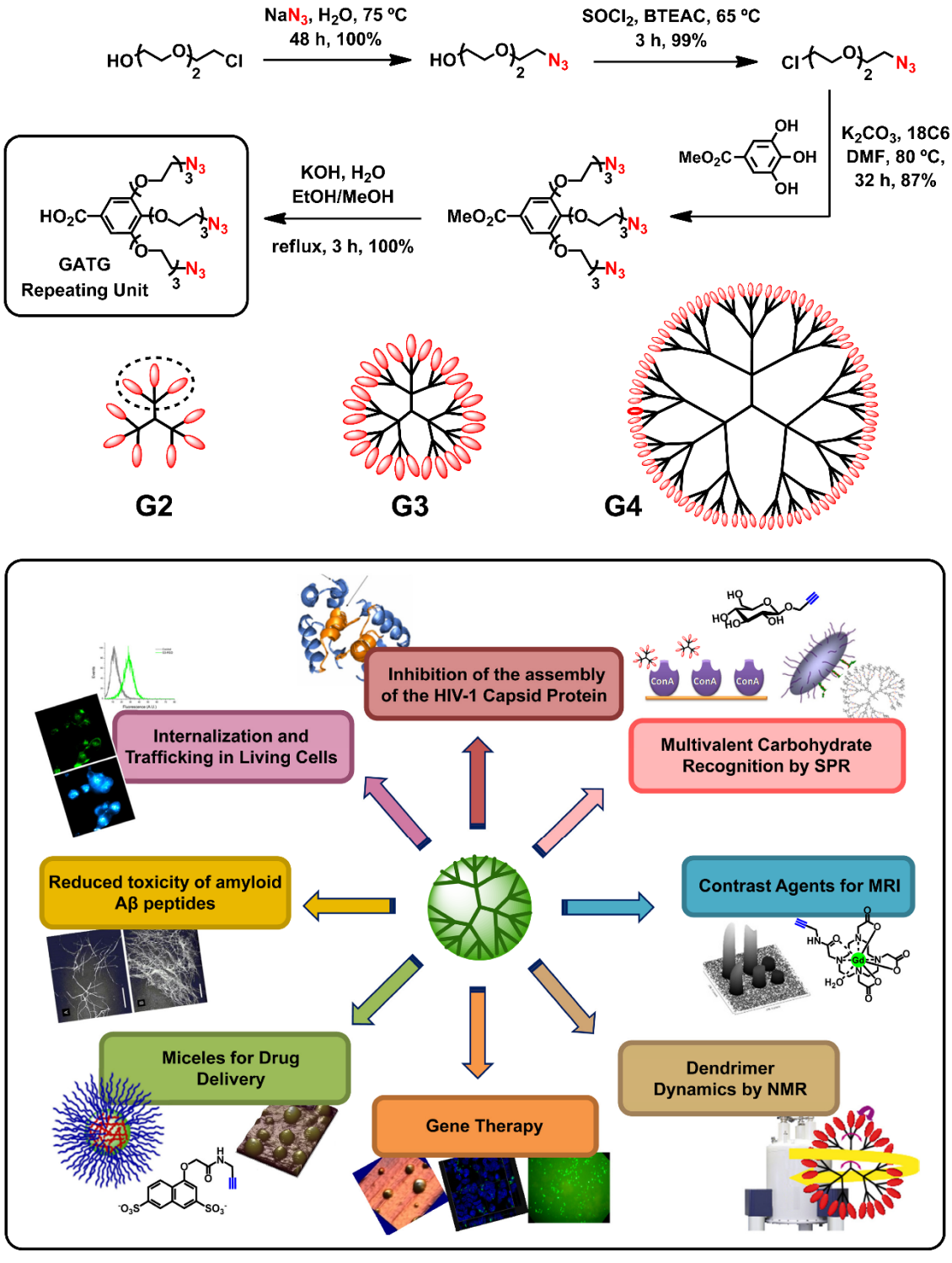


Fig. 1. Structure, synthesis of the repeating unit, and applications of GATG dendrimers. Reprinted with permission from ref. (33).

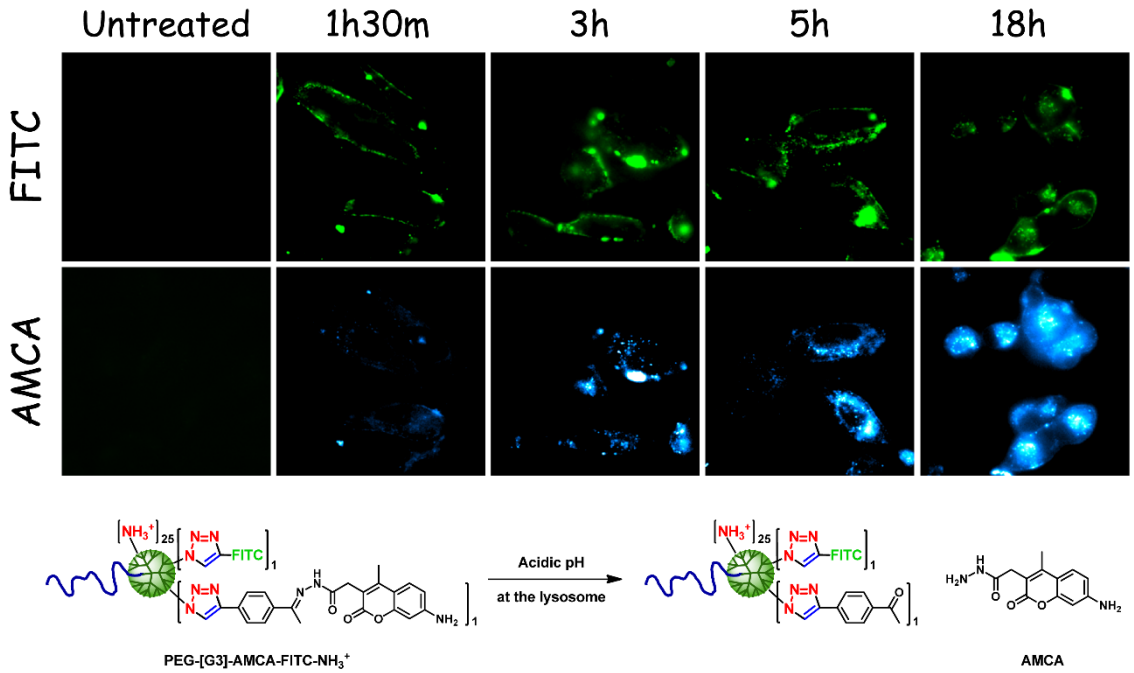


Fig. 2. Schematic structure of PEG-[G3]-AMCA-FITC-NH_3^+. Cell-uptake and intracellular trafficking in HeLa cells: fluorescent signals from FITC (carrier) and AMCA (model cargo). Reprinted with permission from ref. (39).

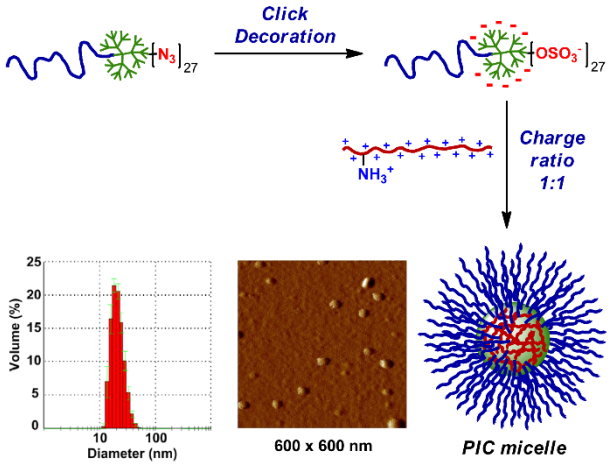


Fig. 3. Schematic representation of the formation of PIC micelles from an anionic PEG-GATG block copolymer and PLL. DLS histogram and tapping-mode AFM image of the micelles. Reprinted with permission from ref. (44).

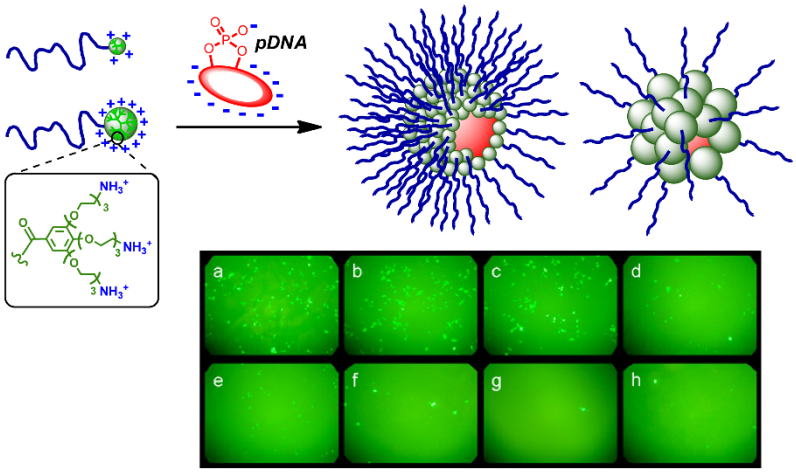


Fig. 4 Schematic representation of dendriplexes prepared from a plasmid DNA (pDNA) and two generations of PEG-GATG block copolymers as nanostructures with core–shell stoichiometry. Expression of EGFP in HEK-293T cells transfected with dendriplexes prepared with increasing molar ratios of PEG-[G3]-NH_2/[G3]-NH_2: (a) 0% (solely G3), (b) 0.5%, (c) 1%, (d) 5%, (e) 10%, (f) 20%, (g) 50% and (h) 100% (solely PEG-G3). Reprinted with permission from ref. (34) and (50).

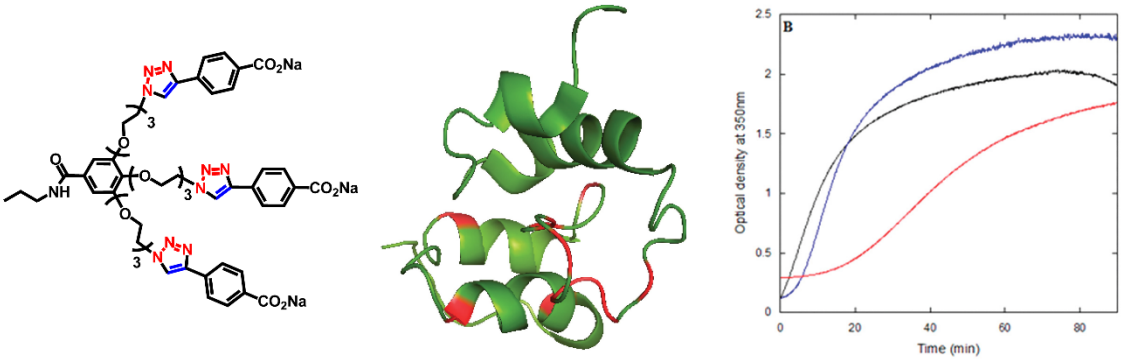


Fig. 5. Left: structure of [G1]-CO_2Na. Centre: binding of GATG dendrimers to monomeric CTDW184A by NMR (residues in red change their peak intensities in the presence of GATG). Right: inhibitory activity of [G1]-CO_2Na on the *in vitro* assembly of CA. Oligomerization of CA in the absence (black line) or presence of [G1]-CO_2Na at a 5-fold (blue line) and 10-fold molar excess (red line). Reprinted with permission from ref. (65).

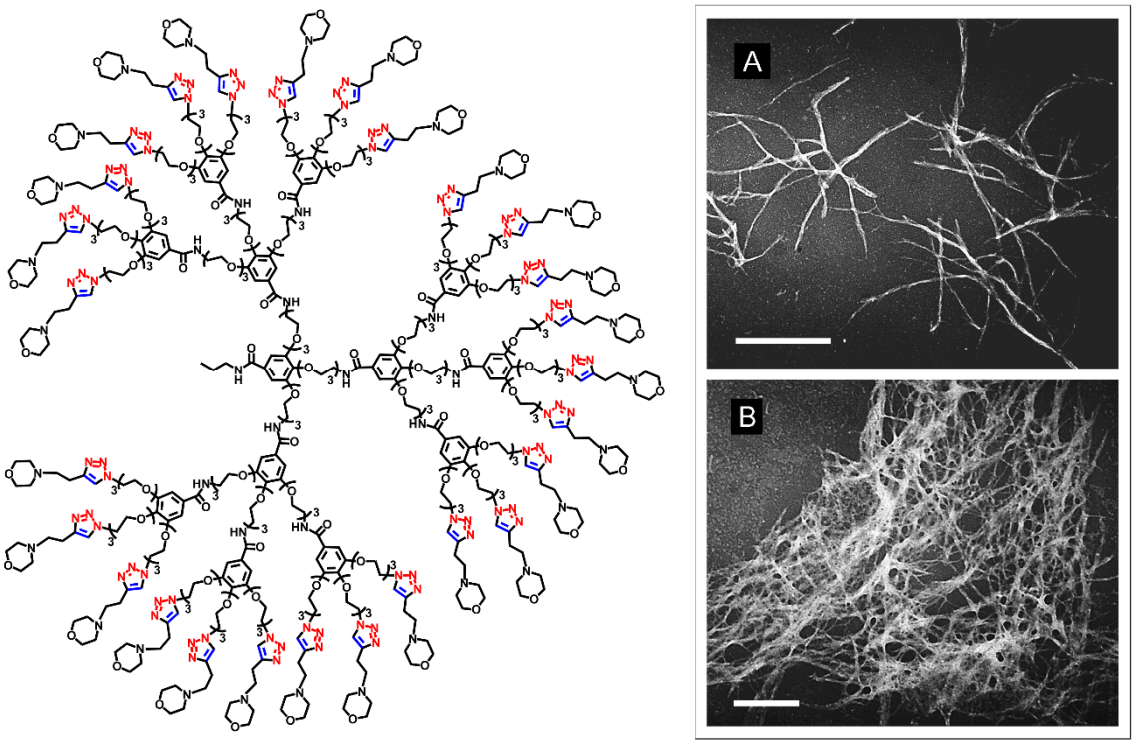


Fig. 6. Left: structure of [G3]-Mor. Right: electron micrographs of Aβ 1-28 at the end of an aggregation process in the absence (A) and the presence of 1 μM [G3]-Mor (B). The length of the bar equals to 200 nm. Reprinted with permission from ref. (69).

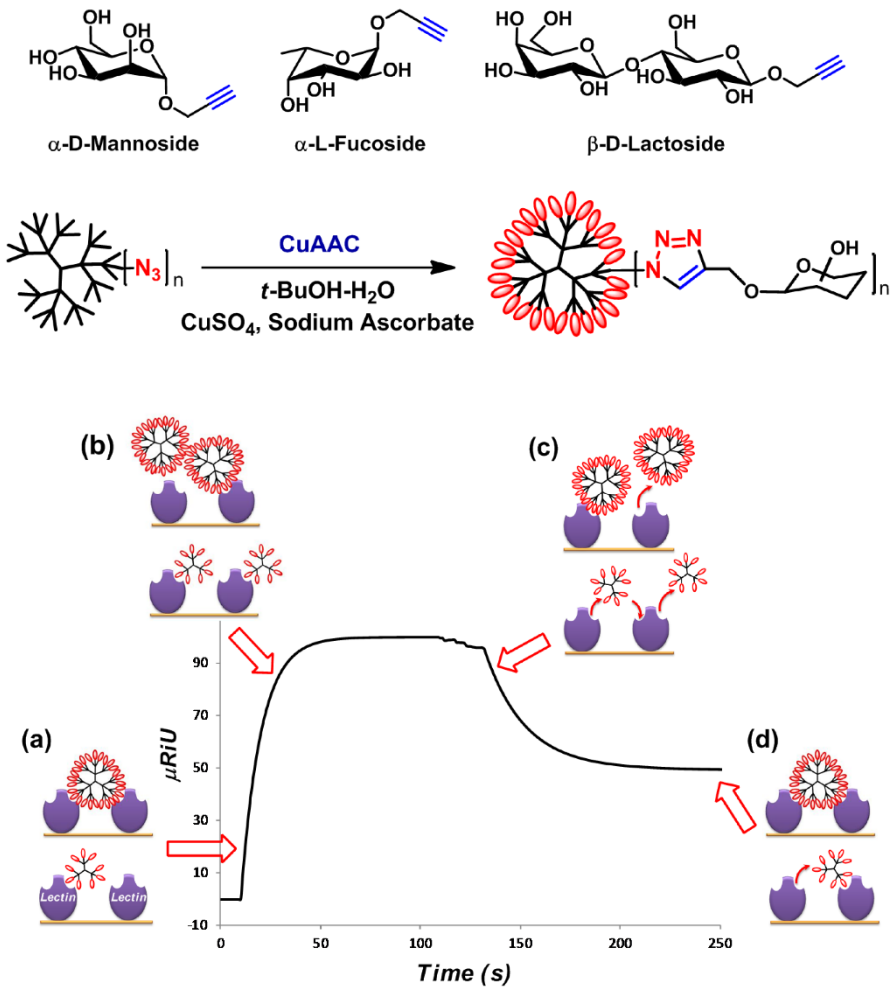
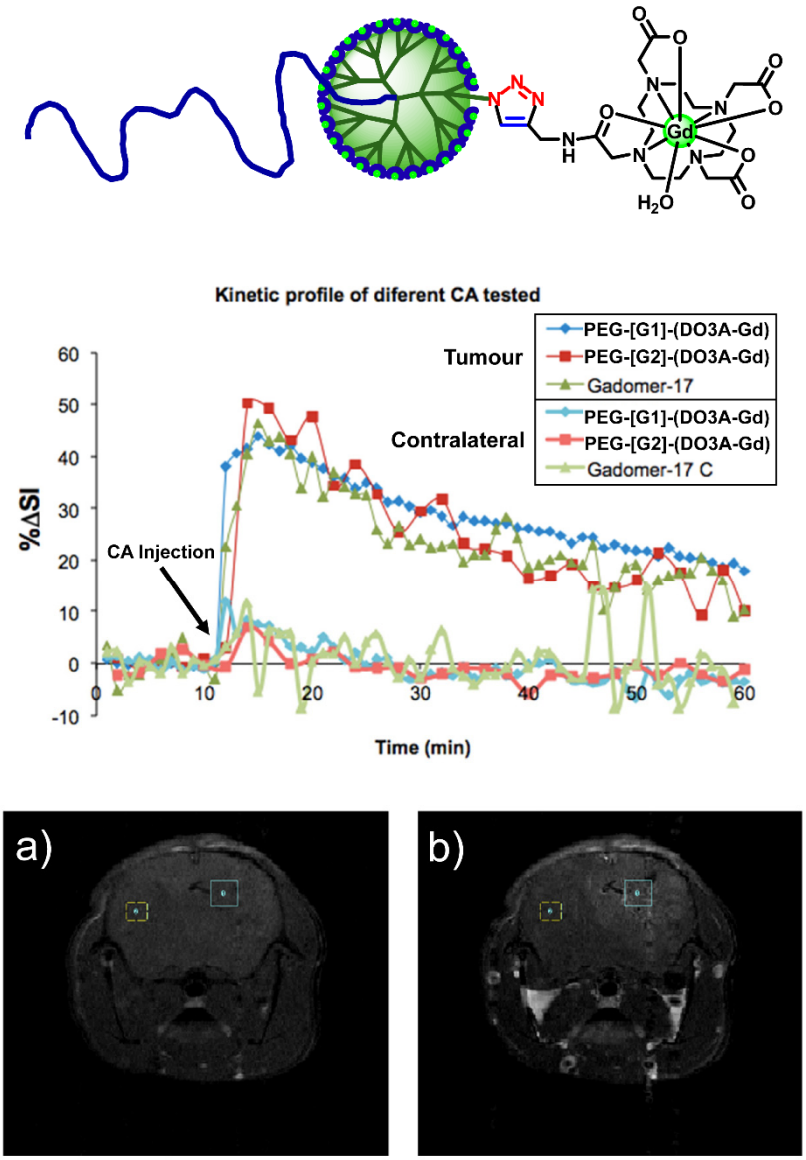


Fig. 7. Synthesis of GATG glycodendrimers and schematic representation of the dynamic binding heterogeneity of surface-bound experiments between lectin clusters and glycodendrimers: (a) Initial binding of glycodendrimers to the lectin cluster with potential stabilization *via* chelate mechanism depending on glycodendrimer size and lectin cluster density. (b) At longer association times, competition between dendrimers for lectin complexation increases, promoting monovalent interactions primarily stabilized *via* rebinding effects. (c) An initial fast dissociation of glycodendrimers bound with low affinity is followed by (d) a slower dissociation due to stabilization by rebinding and potential chelate effects. Reprinted with permission from ref. (32) and (75).



455

456 **Fig. 8.** Schematic structure of PEG-GATG contrast agents for MRI. Normalized increase in
457 signal intensity (ΔSI) in tumor and contralateral hemisphere. T_1-weighted images of a mouse
458 brain before (a) and after (b) administration of PEG-[G2]-(DO3A-Gd). Squares in images show
459 tumor (right) and contralateral hemisphere (left) regions analyzed. Reprinted with permission
460 from ref. (81).

461

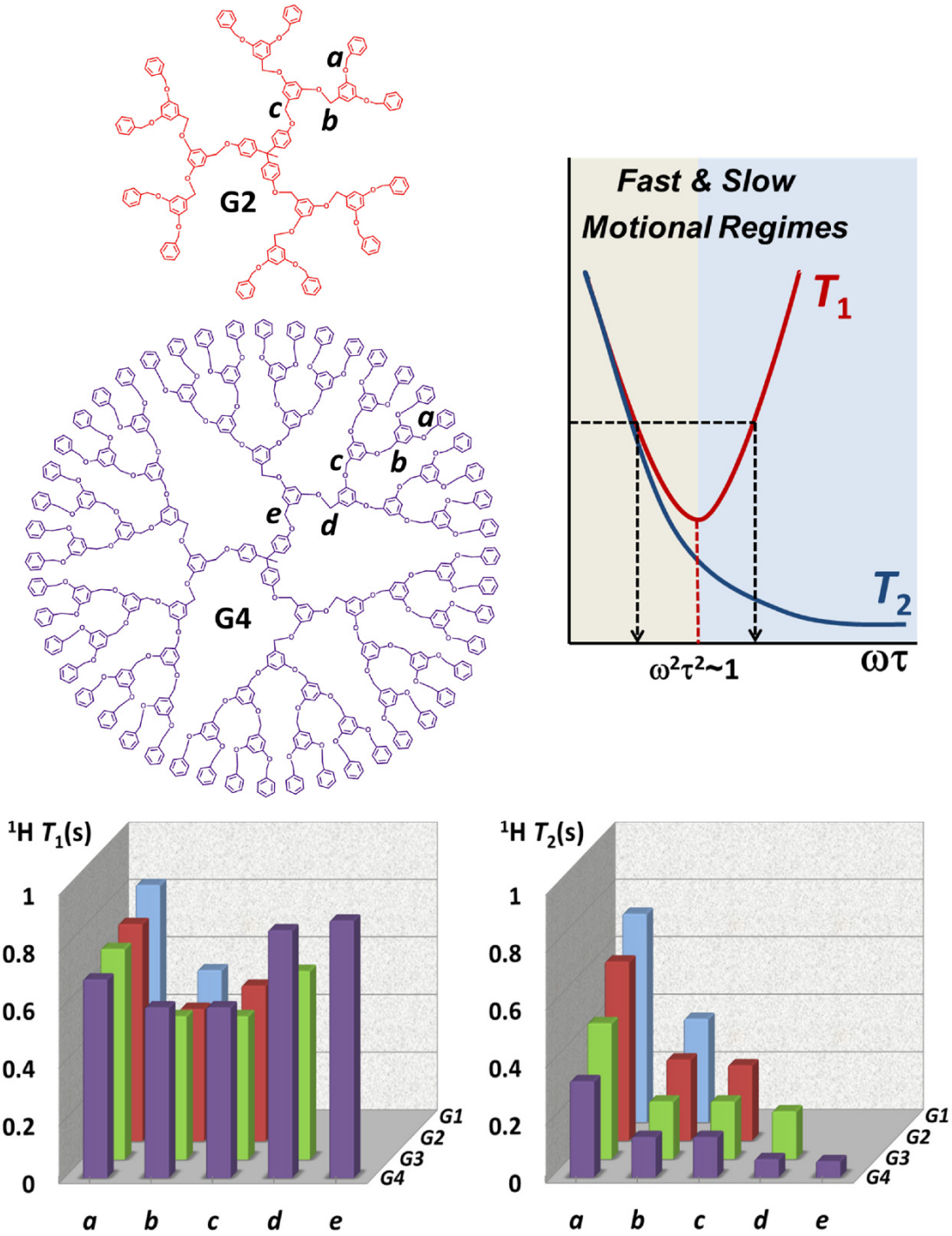


Fig. 9. Top left panel: structures of poly(aryl ether) dendrimers. Top right panel: schematic representation of the theoretical dependence of T_1 and T_2 on correlation time (τ). Bottom panel: ^{1}H T_1 and T_2 for the benzylic protons of G1–G4 poly(aryl ether) dendrimers ($CDCl_3$, 500 MHz, 298 K). Reprinted with permission from ref. (92).

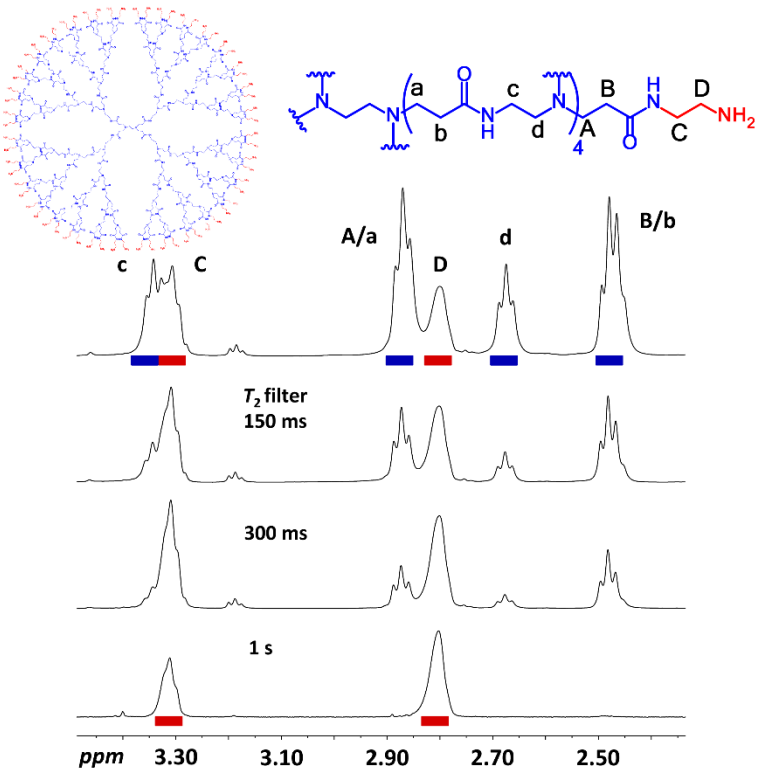


Fig. 10. Structure of G4 PAMAM and ^{1}H T_2-filtered NMR spectra (500 MHz, $CDCl_3$, 298 K). Increasing filters between 150 ms and 1 s resulted in a spectrum showing only the most peripheral protons, which remained partially hidden in the original spectrum. Reprinted with permission from ref. (94).

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