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Self-Assembling Supramolecular Dendrimers for Biomedical Applications: Lessons Learned from Poly(amidoamine) Dendrimers

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CONSPECTUS: Dendrimers, notable for their well-defined radial structures with numerous terminal functionalities, hold great promise for biomedical applications such as drug delivery, diagnostics, and therapeutics. However, their translation into clinical use has been greatly impeded by their challenging stepwise synthesis and difficult purification.

To circumvent these obstacles, we have pioneered a self-assembly approach to constructing noncovalent supramolecular dendrimers using small amphiphilic dendrimer building units which can be easily synthesized and purified. By virtue of their amphipathic nature, the small amphiphilic dendrimers are able to selfassemble and generate large supramolecular dendrimers via noncovalent weak interactions such as van der Waals forces, H bonds, and electrostatic interactions. The so-created noncovalent dendrimers can mimic covalent dendrimers not only in terms of the radial structural feature emanating from a central core but also in



their capacity to deliver drugs and imaging agents for biomedical applications. The noncovalent supramolecular dendrimers can be easily synthesized and modulated with regard to size, shape, and properties by varying the nature of the hydrophobic and hydrophilic entities as well as the dendrimer generation and terminal functionalities, ensuring their adaptability to specific applications. In particular, the dendritic structure of the amphiphilic building units permits the creation of large void spaces within the formed supramolecular dendrimers for the physical encapsulation of drugs, while the large number of surface functionalities can be exploited for both physical and chemical conjugation of pharmaceutic agents for drug delivery.

Poly(amidoamine) (PAMAM) dendrimers are the most intensively studied for biomedical applications by virtue of their excellent biocompatibility imparted by their peptide-mimicking amide backbones and numerous interior and terminal amine functionalities. We present a short overview of our self-assembly strategy for constructing supramolecular PAMAM dendrimers for biomedical applications. Specifically, we start with the introduction of dendrimers and their synthesis, focusing on the innovative self-assembly synthesis of supramolecular dendrimers. We then detail the representative examples of the noncovalent supramolecular PAMAM dendrimers established in our group for the delivery of anticancer drugs, nucleic acid therapeutics, and imaging agents, either within the dendrimer interior or at the dendrimer terminals on the surface. Some of the supramolecular dendrimer nanosystems exhibit outstanding performance, excelling the corresponding clinical anticancer therapeutics and imaging agents. This self-assembly approach to creating supramolecular dendrimers is completely novel in concept yet easy to implement in practice, offering a fresh perspective for exploiting the advantageous features of dendrimers in biomedical applications.

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Figure 1. (A) Chemical structure of the first poly(amidoamine) (PAMAM) dendrimer (1). For clarity, only the dendrimer of generation 3 is presented. (B) Cartoon illustration of a dendrimer comprising a central core or generation 0 (G0), repeating branching units forming consecutive levels or generations (G1, G2, and G3), and the terminal groups on the dendrimer surface.⁹ Adapted with permission from ref 9 (copyright 2018 Science China Press).

Peng, L. Self-assembling supramolecular dendrimer nanosystem for PET imaging of tumors. *Proc. Natl. Acad. Sci. U. S. A.* **2018**, 115 (45), 11454–11459.² *This is the first report on the self-assembly of a small amphiphilic dendrimer to generate a supramolecular dendrimer for tumor imaging using positron emission tomography (PET), outperforming FDG, the clinical gold reference for PET imaging of a tumor.*

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- Liu, X.; Zhou, J.; Yu, T.; Chen, C.; Cheng, Q.; Sengupta, K.; Huang, Y.; Li, H.; Liu, C.; Wang, Y.; Posocco, P.; Wang, M.; Cui, Q.; Giorgio, S.; Fermeglia, M.; Qu, F.; Pricl, S.; Shi, X.; Liang, Z.; Rocchi, P.; Rossi, J. J.; Peng, L. Adaptive amphiphilic dendrimer-based nanoassemblies as robust and versatile siRNA delivery systems. Angew. Chem., Int. Ed. 2014, 53 (44), 11822-11827.⁵ This paper describes an adaptive amphiphilic dendrimer

and its self-assembly into supramolecular systems for the delivery of small interfering RNA (siRNA) in vitro and in vivo.

1. INTRODUCTION

The first dendritic molecules, which are also called "cascade molecules",⁶ were reported by Vögtle et al. in 1978. In 1985, Tomalia et al. synthesized poly(amidoamine) (PAMAM) dendrimers (Figure 1A),⁷ which constituted the first complete dendrimer family. This marked the beginning of the dendrimer era and spurred unprecedented interest among the scientific community in all aspects of dendrimer science, including chemical synthesis, structural characterization, functional evaluation, and various applications.⁸

By definition, a dendrimer is a synthetic molecule with a unique architecture bearing regular cascade-branched repeating units emanating from a focal point and numerous terminal groups on its surface (Figure 1). From a structural perspective, dendrimers comprise three distinct parts: (1) a central core, where dendrimer growth begins; (2) repeated branching units that allow radially organized growth into consecutive layers or generations; and (3) numerous terminals, which can carry a large variety of functional groups and are exposed on the surface of the molecule (Figure 1B). These structural features endow dendrimers with unique structural properties and multivalent cooperativity and also distinguish them from the three traditional architectural categories of polymers, i.e., linear, cross-linked, and simple branched; dendrimers belong to the fourth major architectural class of polymers.¹⁰

By virtue of their well-defined structure and multivalent cooperativity, dendrimers are particularly promising candidates for a wide variety of biomedical applications, such as drug delivery, diagnostics, and therapeutics for treating various diseases.^{11–13} However, their successful implementation in clinical applications would require ready availability of the desired quantity with consistent levels of quality.¹⁴ Unfortunately, stepwise dendrimer synthesis is onerous, and the risk of

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Figure 2. Cartoon representations of (A) the self-assembly of a small amphiphilic dendrimer into a supramolecular dendrimer that mimics a covalently constructed dendrimer.¹ (B) Delivery of hydrophobic and hydrophilic pharmaceutical agents as well as negatively charged nucleic acid therapeutics. Adapted with permission from ref 1 (copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA).

structural defects increases with the number of generations. Such defective dendrimers have similar chemical compositions and physical properties to those of intact dendrimers, which makes dendrimer purification extremely difficult. This important drawback has resulted in the failure of a preclinical trial on the dendrimer-based delivery of an anticancer drug.¹⁵ Despite the recent successful clinical trials of VivaGel and DEP,¹⁶ which are dendrimer-based drugs for the treatment of bacterial vaginosis and cancer, respectively, dendrimer synthesis, particularly of high-generation dendrimers, remains problematic.

To overcome these problems, we designed and spearheaded supramolecular dendrimer synthesis via the noncovalent self-assembly of small amphiphilic dendritic components, whereby hydrophobic interactions, hydrogen bonds, and the hydrophilic/hydrophobic balance all play important roles (Figure 2A).^{1–5,17} This approach is easy to implement in practice and circumvents laborious covalent dendrimer synthesis. The resulting noncovalent supramolecular dendrimers mimic covalent dendrimers in their dendritic structures (i.e., a core, branching units, and terminal groups (Figure 2A)) and also in their capacity for drug delivery in biomedical applications (Figure 2B).

In particular, the dendritic structure of the amphiphilic building units permits the creation of ample voids within noncovalent supramolecular dendrimers for the physical encapsulation of drug molecules with high loading capacities. Furthermore, the numerous surface functionalities can be exploited for the physical and chemical conjugation of therapeutic agents for drug delivery in biomedical applications (Figure 2B). These noncovalently constructed supramolecular dendrimers are easy to prepare. Moreover, their size, shape, and properties can be altered by modifying the hydrophobic and hydrophilic entities, adjusting the dendrimer generation, and changing the terminal groups, thereby ensuring adaptability to specific applications. By employing different small and simple amphiphilic building units, we have successfully constructed various supramolecular dendrimers for the delivery of drugs, nucleic acid therapeutics, and bioimaging agents.^{1-5,18-27}

It is worth mentioning that amphiphilic dendrimers can also readily self-assemble into vesicle structures known as "dendrimersomes".^{28,29} The dendrimersome concept, which differs from the supramolecular dendrimer concept described in the present work, was inaugurated by Percec et al.²⁸ and has been investigated extensively as a means of mimicking membranes in biological applications. State-of-the-art dendrimersomes have been reviewed elsewhere²⁹ and will not be included in this account.

In the present account, we will give a brief overview of the supramolecular dendrimers developed by our group. We will begin by providing a general introduction to the common strategies used for dendrimer synthesis. We will then describe the synthesis of supramolecular dendrimers and highlight their biomedical applications with representative examples that demonstrate their advantages and limitations. It should be noted that, in this account, the term "supramolecular dendrimers" refers to noncovalently constructed dendrimers that have the same structural features as covalent dendrimers (i.e., a central core, regular branching units, and terminal groups). We will not include traditional covalent dendrimers that form supramolecular assemblies without dendritic structures.

2. DENDRIMER SYNTHESIS

Conventional dendrimer synthesis can be achieved using either a divergent (from a multifunctional central core and extending outward; Figure 3A) or a convergent (inward-oriented

A Divergent synthesis







D Supramolecular self-assembly dendrimer



Figure 3. Cartoon illustration of the different approaches to dendrimer synthesis: (A) the divergent approach, (B) the convergent approach, (C) the combined divergent and convergent approach for covalent dendrimer synthesis, and (D) the self-assembly approach for constructing noncovalent supramolecular dendrimers.¹⁷ Adapted with permission from ref 17 (copyright 2019 Elsevier).

synthesis from the dendrimer surface toward a central core; Figure 3B) approach or a combination of the two (Figure 3C).^{8,30} The combined divergent/convergent approach, which is also called the double-stage convergent approach, involves the synthesis of building blocks via a divergent approach followed by convergent dendrimer assembly (Figure 3C). In all of these approaches, the dendrimers are constructed via stepwise covalent synthesis, which is burdensome and arduous. In particular, as the dendrimer grows, structural defects become more common owing to incomplete reactions or side reactions caused by increasing steric hindrance, which reduces reactivity. It is difficult to separate the defective dendrimers from the intact dendrimers because they have very similar chemical and physical properties. Therefore, dendrimer purification is also very difficult and worrisome.

To circumvent the limitations related to dendrimer synthesis via covalent means, we have pioneered the noncovalent synthesis of supramolecular dendrimers via the self-assembly of small amphiphilic dendritic components, which can be synthesized readily in pure form (Figure 3D).^{1-3,5,18,19,21-24} Self-assembly relies on the cumulative effects of multiple

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noncovalent interactions to assemble small molecular building blocks into supramolecular assemblies with weak interactions, such as van der Waals forces, hydrogen bonds, and electrostatic interactions, in a reversible, controllable, and specific way.^{31,32} The noncovalent supramolecular dendrimers formed by selfassembly have the same structural features as covalent dendrimers (i.e., a distinctive core, repetitive branching units, and terminal groups). The concept of supramolecular dendrimer synthesis via self-assembly is new; it requires relatively little synthesis effort yet offers new and specific properties for related applications.^{17,33} Using PAMAM dendrimers as examples, we describe herein our recent work on the self-assembling synthesis of supramolecular dendrimers for biomedical applications, such as drug delivery, nucleic acid therapeutics, and imaging agents.

3. PAMAM DENDRIMERS

Since the seminal report by Tomalia et al.,7 PAMAM dendrimers have been the most intensively studied of all dendrimers, and some are also commercially available. With numerous amide and tertiary amine functionalities in their interiors and primary amine terminals on their surfaces, the very first PAMAM dendrimers developed by Tomalia et al. were originally envisioned to mimic proteins.⁷ These peptidemimicking features endow PAMAM dendrimers with high water solubility and excellent biocompatibility, which explains their frequent use and their potential for various biomedical applications compared with other dendrimers.^{12,34}

For example, we conceived and synthesized a structurally flexible PAMAM dendrimer bearing a triethanolamine (TEA) $core^{35}$ (Figure 4) to mimic the natural protein histone for DNA binding and DNA delivery.³⁶ The TEA constitutes an extended core offering ample space within the dendrimer to accommodate the branching units with structural flexibility. The positively charged terminal amine groups can interact electrostatically with the negatively charged nucleic acids, thereby protecting them from degradation and enabling their delivery to the desired destination.³⁰

High-generation TEA-core dendrimers are particularly useful for delivering nucleic acid molecules such as DNA, small interfering RNA (siRNA), and small activation RNA (saRNA) for gene therapy in various disease models, both *in* vitro and *in* vivo.³⁷⁻⁴⁶ Our TEA-core fifth-generation dendrimer was scheduled to undergo clinical trials for the delivery of anticancer saRNA therapeutics to treat advanced liver cancer.44 Unfortunately, because it fell short of the demands of good manufacturing practice (GMP), it was eventually substituted with a lipid vector formulation (Smarticles),⁴⁷ the saRNA delivery performance of which is largely inferior to that of the TEA-core dendrimer. This definitely constitutes an unsuccessful example of clinical translation, although in general dendrimers have great potential for use in biomedical applications. However, their synthesis does present a significant challenge.¹²

4. SELF-ASSEMBLING SUPRAMOLECULAR PAMAM DENDRIMERS

We pioneered the self-assembly approach to constructing noncovalent supramolecular dendrimers to overcome the obstacles to implementing PAMAM dendrimers for biomedical applications.^{1-5,18-27} Small amphiphilic dendrimers bearing hydrophobic alkyl chains and hydrophilic PAMAM dendrons



Figure 4. Chemical structure of a poly(amidoamine) (PAMAM) dendrimer (2) bearing a triethanolamine (TEA) core developed by our group. Only the third-generation dendrimer is shown.³⁵ Adapted with permission from ref 35 (copyright 2005 Royal Society of Chemistry).

can be readily synthesized in large quantities with high purity using either divergent or convergent methods or a combination of both.^{1,2,5,18-24} By virtue of their amphipathic nature, the hydrophobic interactions in their core regions, and the hydrogen bonds within their dendron shells, these small amphiphilic dendrimers can self-assemble into stable and robust noncovalent supramolecular dendrimers. It is easy to modify the length and nature of the hydrophobic chain and/or the generation of the hydrophilic PAMAM dendron and to alter the different functionalities of the terminals. These changes provide incomparable structural flexibility and diversity for a wide range of potential biomedical applications. The following report is a brief summary of our studies in this direction. We present and discuss the promising breakthroughs and challenges faced using representative examples of the delivery of nucleic acid therapeutics, anticancer drugs, and imaging agents.

4.1. Nucleic Acid Delivery

Nucleic acids have emerged as novel therapeutic agents for the treatment of various diseases, particularly since the discovery of RNA interference (RNAi).⁴⁸ RNAi involves the silencing of a targeted gene with siRNA, which sequence-specifically pairs with, and subsequently cleaves, the corresponding mRNA. The challenge facing siRNA therapeutics is delivery because siRNA is not stable and can be easily degraded. Furthermore, the negative charge and strong hydrophilicity of siRNA prevent it from crossing cell membranes readily. Moreover, at high concentrations, naked siRNA can generate unwanted and sometimes serious adverse effects. Consequently, suitable delivery systems that are able to prevent siRNA degradation,

mask its negative charge, and ensure its safe and effective delivery to target cells for functional gene silencing are critical. 49

We have been actively engaged in developing dendrimer nanovectors for siRNA delivery. Figure 5 illustrates the first amphiphilic dendrimer (3) and its derivatives (4 and 5), which were developed by our group to construct supramolecular dendrimers for siRNA delivery.^{1,20–22} Each dendrimer bears a long hydrophobic alkyl chain of 18 carbon atoms and a small hydrophilic PAMAM dendron with terminals of primary amines, tertiary amines, and arginine residues (Figure 5A). They self-assemble into supramolecular dendrimers by virtue of their amphiphilicity and form spherical nanomicelles to mimic covalent dendrimers. They can interact with the negatively charged siRNA via their positively charged terminals for siRNA binding and cellular uptake, followed by endosomal release via the "proton-sponge" effect for potent gene silencing (Figure 5B). Figure 5C shows the transmission electron microscope (TEM) images of the supramolecular dendrimer formed with 3^{1} , which is similar in size to the high-generation TEA-core covalent PAMAM dendrimers. Most importantly, this supramolecular dendrimer can mimic TEA-core fifthgeneration dendrimer 2 to deliver siRNA, with potent gene silencing at both the mRNA and protein levels (Figure 5D,E) for effective anticancer activity in RNAi-based cancer treatment. The synthesis of 3 is much easier than that of TEA-core fifth-generation dendrimer 2 (Figure 4), and the creation of a supramolecular dendrimer comprising 3 is spontaneous in water.^{1,21} Our studies on dendrimer 3 constitute the exquisite demonstration of a supramolecular dendrimer created by self-



Figure 5. Amphiphilic dendrimers 3-5 and their self-assembly into supramolecular dendrimers for small interfering RNA (siRNA) delivery. (A) Chemical structures of 3-5 with primary amine, tertiary amine, and arginine residue terminals, respectively. (B) Cartoon illustration of the self-assembly of an amphiphilic dendrimer into a supramolecular dendrimer for siRNA binding and delivery, including cell uptake, endosome release, and subsequent gene silencing. (C) Transmission electron microscope (TEM) images of the supramolecular dendrimer formed by 3. Gene silencing of Hsp27 at the mRNA level (D) and the protein level (E) in tumor-xenograft mice following treatment with siRNA delivered by a 3-assembled supramolecular dendrimer. (F) Toxicity profile of 4 in human embryonic kidney 293 (HEK293) cells compared with that of 3. (G) Reduced expression of oncogene Myc at the protein level in patient-derived cancer organoids following treatment with 4, Myc siRNA, and Myc siRNA delivered by commercial vector Lipofectamine (Lipo) and by the supramolecular dendrimer formed with 4. (H) Cell uptake of siRNA delivered by the supramolecular dendrimer formed with 5 (left) was visibly higher than that delivered by the supramolecular dendrimer formed with 3 (right).^{1,20-22} Reproduced and adapted with permission from ref 1 (copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA), and ref 22 (copyright 2020 Tsinghua University Press and Springer), ref 21 (copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA), and ref 22 (copyright 2015 Royal Society of Chemistry).

assembling a small amphiphilic dendrimer. Such a dendrimer is able to mimic a covalently synthesized high-generation dendrimer in terms of size, shape, and siRNA delivery capability.

It is worth noting that the supramolecular dendrimer formed with 4 is less toxic than that formed with 3 owing to the stronger tendency of the tertiary amine terminals toward ionization (Figure 5F).²⁰ Furthermore, it effectively delivers siRNA to silence oncogene Myc in patent-derived tumor organoids (Figure 5G) and has anticancer activity that exceeds that of gold standard vector Lipofectamine 2000. Dendrimer 5 is able to mimic cell-penetrating peptides to promote cellular uptake²² because the positively charged guanidinium terminals alongside the amine groups in the arginine residues are able to form divalent hydrogen bonds and electrostatic interactions with the negatively charged groups on the cell surface, thereby facilitating membrane penetration. Compared with non-

arginine dendrimer 3, the supramolecular dendrimer formed from 5 allows considerably enhanced cellular uptake of siRNA (Figure 5H), resulting in much more potent gene silencing. These studies demonstrate that simply by altering their terminal functionalities it is possible to impart the specific activity and desired safety profile of the supramolecular dendrimers for nucleic acid delivery.

To investigate the impact of the amphiphilicity and hydrophobicity/hydrophilicity balance in self-assembled supramolecular dendrimers for siRNA delivery, we studied amphiphilic dendrimers 3a-e derived from 3 with varying alkyl chain lengths, dendron 6 without any chain, and dendrimer 7 with a hydrophilic oligo(ethylene glycol) chain (Figure 6).^{1,21} Both 6 and 7 lack amphiphilicity and are therefore unable to self-assemble into the supramolecular structure needed for siRNA delivery. This highlights the importance of the amphiphilicity of 3 and its ability to self-



Figure 6. (A) Chemical structures of amphiphilic dendrimers 3a-e derived from 3 with alkyl chains of differing lengths, dendron 6 without any chain, and dendrimer 7 bearing a hydrophilic oligo(ethylene glycol) chain. (B) Transmission electron microscope (TEM) images of the supramolecular dendrimers formed by amphiphilic dendrimers 3c-e with different chain lengths.^{1,21} Reproduced and adapted with permission from ref 1 (copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA) and ref 21 (copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA).



Figure 7. Amphiphilic dendrimer 8 and its self-assembling supramolecular structures for small interfering RNA (siRNA) delivery and gene silencing. (A) Chemical structure of 8 and cartoon illustration of the self-assembly of 8 into its supramolecular dendrimersome structure before rearrangement into nanomicelles following siRNA binding for siRNA delivery. (B) Transmission electron microscope (TEM) images of the dendrimersome assembled from 8. (C) TEM images of the siRNA/8 complexes in which the dendrimersomes formed from 8 have rearranged into supramolecular dendrimer nanomicelles following interaction with the negatively charged siRNA. (D) Tat/rev mRNA expression in T cells. (E) NKG2D expression in natural killer cells. (F) JAK1 protein expression in macrophages following treatment with the corresponding siRNA molecules delivered by 8, compared with the nontreatment control, the dendrimer alone, or scrambled siRNA delivered by $8.^{5,24}$ Reproduced and adapted with permission from ref 5 (copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA) and ref 24 (copyright 2020 Springer Nature).



Figure 8. Targeted small interfering RNA (siRNA) delivery and gene silencing in cancer treatment using amphiphilic dendrimer 8. (A) Cartoon illustration of the siRNA/8 complex decoration with targeting peptide $E_{16}G_6RGDK$ for targeted delivery. (B) Enhanced downregulation of target gene Hsp27 at the mRNA level by siRNA/8/ $E_{16}G_6RGDK$ is reduced in the presence of either cRGD or anti-Nrp-1-receptor antibody. (C) Tumor growth in xenograft mice treated with phosphate-buffered saline (PBS), siRNA/8, and siRNA/8/ $E_{16}G_6RGDK$.⁴ Reproduced and adapted with permission from ref 4 (copyright 2018 American Chemical Society).

assemble into a robust supramolecular dendrimer structure, which enables it to mimic a covalent high-generation dendrimer with multivalent cooperativity for effective siRNA delivery.²¹ It is also important to note that dendrimers **3a** and **3b** with short alkyl chains of C12 and C14 fail to form stable supramolecular dendrimers owing to insufficient hydrophobic interactions with the short alkyl chains, whereas the dendrimers with the corresponding hydrophobic chains of C16, C18, C20, and C22 generate stable supramolecular dendrimer nanomicelles with sizes increasing from 6.8 to 7.8 nm (Figures 6B and 5C).¹ Taken together, these studies underline the impact of the alkyl chain length and the balance of amphiphilicity on the formation and size of stable supramolecular dendrimers.

We further modified the hydrophobic part of 3 by appending two hydrophobic alkyl chains to synthesize dendrimer 8 (Figure 7).⁵ Remarkably, 8 can spontaneously self-assemble into vesicle-like dendrimersomes (Figure 7B) instead of the supramolecular dendrimers with micellar structures formed by 3. Most interestingly, upon interaction with siRNA, the vesicle-like dendrimersomes formed by 8 are able to undergo concurrent structural rearrangement into small, spherical micelles (Figure 7C). This graceful vesicularto-micellar structural transition allows 8 to maximally expose its positively charged terminal amine groups, ensuring stronger electrostatic interactions with the negatively charged siRNA. This enables the effective entrapment and condensing of siRNA into stable nanoparticles, thereby sheltering it from degradation before delivery to the desired destination. In addition, this dendrimer is able to capitalize on the delivery advantages of both lipid and dendrimer vectors. This allows it to effectively deliver siRNA to a variety of different cell types including primary and stem cells as well as immune cells such as primary T cells (Figure 7D), natural killer cells (Figure 7E),

and macrophages (Figure 7F), which are otherwise highly challenging for nucleic acid delivery. 5,24-26

To promote targeted delivery, we further established a supramolecular dendrimer delivery system based on 8 using a peptide harboring dual-targeting warhead RGDK (Figure 8).⁴ RGDK is able to target tumor vasculature via the binding of the arginine-glycine-aspartic acid peptide (RGD) to the overexpressed integrin while simultaneously enhancing cell penetration via RGDK binding to neuropilin-1 receptors on the cancer cell surfaces. In practice, the RGDK segment is incorporated at the terminal of the E₁₆G₆RGDK peptide. This peptide comprises a middle portion containing oligo(glycine) G₆, which acts as the linker, and negatively charged oligo(glutamic acid) E_{16} at the opposite terminal, which serves to promote the attachment to the positively charged siRNA/8 complex via electrostatic interaction (Figure 8A). The resulting delivery system enables efficient and specific uptake by cancer cells owing to RGD/integrin and RGDK/neuropilin-1 receptor interaction (Figure 8B), giving rise to much more effective and potent gene silencing and greatly improved anticancer activity (Figure 8C).⁴

The studies described above demonstrate the extraordinary ability of dendrimer 8 to undergo dynamic self-assembly and rearrangement in the presence of siRNA and the targeting peptide to form adaptive supramolecular assemblies. These unique and highly modular features together with ease of synthesis make 8 a promising candidate for the functional delivery of siRNA and other nucleic acid therapeutics.^{4,5,24–26} Collectively, these studies highlight the potential of the self-assembling approach in the construction of modular and functional supramolecular dendrimers for siRNA delivery and gene silencing in biomedical applications.



Figure 9. Self-assembling supramolecular dendrimer for drug encapsulation and drug delivery in cancer therapy. (A) Cartoon representation of the self-assembly of amphiphilic dendrimer 8 into supramolecular dendrimer nanomicelles and the encapsulation of anticancer drug doxorubicin (DOX) within its hydrophobic interior. (B) Transmission electron microscope (TEM) images of the DOX-loaded supramolecular dendrimers formed from 8. (C) Fluorescent imaging revealing the accumulation of the supramolecular dendrimers in tumors via the enhanced permeability and retention (EPR) effect. (D) Significant inhibition of tumor growth in mice and (E) mouse survival rate following treatment with the DOX/8 supramolecular dendrimer nanomicelles, compared with the control treatments of phosphate-buffered saline (PBS), dendrimer alone, and DOX alone.³ Reproduced and adapted with permission from ref 3 (copyright 2015 National Academy of Sciences).



Figure 10. Amphiphilic dendrimers 9 and 10 bearing covalently conjugated bioactive nucleosides at the terminals for drug delivery in response to enzyme hydrolysis.²⁷ Adapted with permission from ref 27 (copyright 2018 Royal Society of Chemistry).

4.2. Drug Delivery

The aim of drug delivery is to maximize drug bioavailability to achieve optimal therapeutic efficacy while minimizing adverse effects.⁵⁰ Unlike covalently constructed dendrimers, self-assembled supramolecular dendrimers have a large void within their interior cores, which could potentially encapsulate drug molecules with high loading contents (Figure 2). Therefore, we developed a supramolecular dendrimer system for drug delivery using 8 (Figure 9A) and determined its capacity to

deliver drugs with anticancer activity.³ Remarkably, the ample void within the supramolecular dendrimer formed by 8 is able to effectively and stably encapsulate anticancer drug doxorubicin (DOX) with up to 40% drug-loading efficiency (Figure 9B). The resulting DOX-loaded supramolecular dendrimer accumulates within the tumor lesion owing to the enhanced permeability and retention (EPR) effect (Figure 9C) and enters the cancer cells via macropinocytosis, allowing greatly enhanced cellular uptake while bypassing drug efflux. The



Figure 11. Amphiphilic dendrimers **11, 12,** and **13** bearing radionuclide $[^{111}In]In^{3+}$ or $[^{68}Ga]Ga^{3+}$ chelated to macrocyclic cage 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazetic acid (DOTA) or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) at the dendrimer terminals for self-assembly into supramolecular dendrimer nanoprobes for tumor imaging based on single photon emission computed tomography (SPECT) and positron emission tomography (PET). (A) Chemical structures of **11, 12,** and **13** and a cartoon illustrating their self-assembly into supramolecular dendrimer nanoprobes for tumor imaging based on single photon emission computed tomography (SPECT) and positron emission tomography (PET). (A) Chemical structures of **11, 12,** and **13** and a cartoon illustrating their self-assembly into supramolecular dendrimer probes formed from **11** (left), **12** (middle), and **13** (right). (C) Representative μ SPECT/CT maximum intensity projection images of tumor-xenograft mice 24 h after intravenous injection of the supramolecular dendrimer probes formed from **11** (left) and **12** (right). The orange ovals indicate the tumor positions. (D) Representative PET image of an orthotopic SOJ6-xenograft mouse 2 h after the intravenous injection of **13**. The orange ovals indicate the tumor positions. (E) Tumor uptake of **13** in an SOJ6-xenograft mouse (upper panel) compared with that of $[^{18}F]$ FDG (lower panel), the clinical gold reference for PET imaging in oncology. The orange arrows indicate the tumor positions. 2,18,19 Reproduced and adapted with permission from ref 2 (copyright National Academy of Sciences), ref **18** (copyright 2020 Royal Society of Chemistry), and ref **19** (copyright 2020 Wiley-VCH GmbH).

DOX-loaded supramolecular dendrimer thus significantly increases the therapeutic potency of DOX (Figure 9D), overcomes doxorubicin resistance in various cancer models including drug-resistant cancers, and largely abolishes the toxicity associated with free DOX (Figure 9D,E). These results are extremely encouraging for the future of novel and effective supramolecular dendrimers for drug delivery in cancer therapy.

Amphiphilic dendrimers can also be chemically conjugated with multiple copies of a bioactive molecule at the dendrimer terminals to construct self-assembling supramolecular dendrimers for drug delivery. Therefore, we created amphiphilic dendrimer 9 (Figure 10) by appending bioactive nucleoside derivatives to the dendrimer terminals via biodegradable ester bonds with a view to achieving responsive drug release following enzymatic action.²⁷ Notably, the enzymatic hydrolysis of dendrimer conjugate 9 is considerably slower than that of lower-generation dendrimer 10 (Figure 10). This can be ascribed to enhanced steric hindrance with increasing dendron generation, which leads to reduced hydrolysis. This apparently negative dendritic effect could nevertheless be exploited in the tailored design of dendrimers for generation-dependent controlled drug release.

4.3. Bioimaging

Biomedical imaging has revolutionized disease management by providing precise information for diagnosis and by supporting decision making in therapy.⁵¹ Nanotechnology is expected to further improve the sensitivity and specificity of tumor imaging

by allowing the accommodation of a large number of imaging agents within the nanoprobe while exploiting the EPR effect for passive tumor targeting.^{52,53} We have therefore developed tumor imaging nanoprobes using supramolecular dendrimers formed by the self-assembly of amphiphilic dendrimers.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) have the highest sensitivity among the commonly used noninvasive imaging modalities and they can be used to quantitatively assess functional information for disease diagnosis.⁵¹ Whereas PET offers higher sensitivity and resolution, SPECT is more readily accessible and less expensive for routine use. Figure 11 illustrates amphiphilic dendrimers 11 and 12, which we conceived and developed to construct supramolecular dendrimer nanoprobes for SPECT and PET imaging.^{2,18,19} The first ever supramolecular dendrimer nanoprobe for SPECT imaging was formed from 11.¹⁸ This dendrimer is composed of a hydrophobic C18 alkyl chain and a hydrophilic PAMAM dendron with terminals bearing SPECT reporting units comprising $[^{111}In]In^{3+}$ chelated to a macrocyclic 1,4,7,10tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) ring. The DOTA cage is the "gold standard" chelator for many radionuclides including ¹¹¹In^{3+ 54} Because the DOTA macrocycle is large and to avoid eventual synthesis trouble and vulnerability stemming from possible steric crowding of the dendrimer terminals, we synthesized dendrimer 11, which has only four DOTA cages at its terminals. As expected, 11 self-



Figure 12. Encapsulation of the imaging agent within the interior of the self-assembling supramolecular dendrimer for bioimaging. (A) Cartoon illustration of the self-assembly of amphiphilic dendrimer 8 into the supramolecular dendrimer to encapsulate fluorescent dye DiR for near-infrared fluorescent imaging of the tumor. Fluorescent emission spectra of DiR when (B) encapsulated within the supramolecular dendrimer formed by 8 in water and (C) dissolved in organic solvent MeOH. (D) Transmission electron microscope (TEM) image of the DiR/8 nanoprobe. (E) Representative fluorescent images of tumor-xenograft mice 0.5, 2, 8, 12, 24, and 48 h after intravenous injection of the DiR/8 nanoprobe in comparison with that of DiR and PBS treatment.³ Reproduced with permission from ref 3 (copyright 2015 National Academy of Sciences).

assembled into small and uniform supramolecular dendrimer nanomicelles (Figure 11B, left panel), which accumulated effectively within the tumor via the EPR effect for SPECT imaging (Figure 11C, left panel). The representative SPECT image of a tumor-xenograft mouse obtained using the nanoprobe formed by 11 is presented in Figure 11C on the left, where the orange oval indicates the tumor position. However, this image also reveals considerable uptake in other organs such as the liver.¹⁸

To reduce liver uptake, we developed dendrimer 12, which bears the smaller macrocyclic 1,4,7-triazacyclononane-1,4,7triacetic acid (NOTA) scaffold instead of the DOTA cage as the chelator for $[^{111}In]In^{3+}$ (Figure 11A).¹⁹ The NOTA ring can form a neutral complex when chelating trivalent metal ion In^{3+} (12 in Figure 11), and it also generates more stable complexes with small metal ions such as In^{3+} , Ga^{3+} , and Cu^{2+} as it is smaller than the DOTA cage. Remarkably, the supramolecular dendrimer formed by 12 is similar in size to that formed by 11 (Figure 11B, middle panel) but gives rise to a highly favorable biodistribution profile with drastically reduced uptake by the liver and other organs, permitting a significantly improved tumor imaging contrast (Figure 11C, right panel). Thus, this study provided interesting perspectives for improving the biodistribution of supramolecular dendrimers for bioimaging.

Dendrimer 13 was established to construct supramolecular dendrimer nanoprobes for PET imaging (Figure 11).² We opted for $[{}^{68}Ga]Ga^{3+}$ as the radioactive reporter for PET because it can be conveniently generated for on-site and on-demand production. It is worth noting that the NOTA cage is an excellent chelator for $[{}^{68}Ga]Ga^{3+}$ and has superior thermodynamic stability and kinetic inertness compared with the DOTA ring, owing to the perfect match in terms of size, geometry, and denticity between the NOTA cage and Ga^{3+} .⁵⁴ Dendrimer 13 readily self-assembles into a supramolecular dendrimer measuring 15 nm in diameter (Figure 11B, right panel), which, via the EPR effect, accumulates in the tumor, as highlighted by the orange oval in the representative PET image in Figure 11D.² The nanoprobe formed from 13 also has a very favorable biodistribution. Most importantly, the combination

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of dendrimer multivalence and EPR-mediated passive tumor targeting with supramolecular dendrimers permits superior imaging sensitivity and specificity compared with clinical gold reference [18 F]FDG (2-fluorodeoxyglucose) for PET imaging. It is worth noting that this supramolecular dendrimer nanoprobe enables the detection of tumors (Figure 11E, upper panel) that are undetectable using [18 F]FDG (Figure 11E, lower panel).²

We also built imaging probes by encapsulating the hydrophobic imaging agents within the interior of the supramolecular dendrimers (Figure 12A), in the same manner as for the delivery of anticancer drug DOX.³ For example, fluorescent dye DiR has excellent near-infrared fluorescence (NIRF) properties for bioimaging but is insoluble in water, which largely limits its applications in biological studies. Upon encapsulation within the interior of the supramolecular dendrimer formed by 8, the resulting DiR/8 nanoprobe is readily soluble in water while retaining the NIRF properties of DiR (Figure 12B,C). Furthermore, the DiR/8 nanoprobe is spherical in form with a size of 15 nm (Figure 12D) and accumulates efficiently within tumors via the EPR effect for effective NIRF imaging (Figure 12E). Taken together, our studies demonstrate the promising advantages of selfassembling supramolecular dendrimers, which can be easily constructed, modulated, and adapted for various bioimaging modalities.

5. CONCLUDING REMARKS

This short account was written with the intention of providing out-of-the-box thinking about the construction of supramolecular dendrimers for biomedical applications. Completely different from conventional covalent synthesis, our proposed noncovalent synthesis by the self-assembly of small amphiphilic dendrimer building units is new in concept and easy to implement in practice as well as adaptable and modular with regard to the various properties and functions required for particular applications. We have successfully implemented this approach to create noncovalent dendrimers that are able to mimic covalently constructed dendrimers for the delivery of drugs, nucleic acid therapeutics, and imaging agents using PAMAM dendrimers as examples. PAMAM dendrimers are particularly appealing for biological applications by virtue of their biocompatibility, which arises from their peptidemimicking amide backbones and numerous amine functionalities. The various supramolecular PAMAM dendrimers presented in this account have great potential for biomedical applications such as diagnosis, cancer therapy, gene therapy, and immune therapy. The self-assembly approach to dendrimer synthesis can also be easily applied to other dendrimers in general. It therefore constitutes a new and possibly key conceptional breakthrough and an outside-the-box perspective for the biomedical application of dendrimers.

The translation of supramolecular dendrimers into real clinical interventions can be both promising and challenging. Despite amphiphilic dendrimers being much easier to synthesize than conventional PAMAM dendrimers, the reproducible scale-up of supramolecular dendrimers can be demanding, as with all other nanosystems for biomedical applications.⁵³ The stability of supramolecular dendrimers is also a key concern because the self-assembly process is driven by weak interactions. Interactions with biomolecules in the body or dilution in the blood may cause the disintegration of supramolecular dendrimers, leading to the premature release of

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the encapsulated cargo.⁵⁰ Moreover, as with any foreign particles that enter the body, supramolecular dendrimers may be recognized and eliminated by the reticuloendothelial system, resulting in limited efficacy.^{52,55} All of these obstacles must be circumvented for real clinical implementation. Fortunately, the experience and knowledge gained from the development of lipid- and supramolecular chemistry-based nanomedicine^{50,55} will certainly benefit and accelerate the translation of supramolecular dendrimer nanosystems. We are actively pursuing this direction of research.

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ABBREVIATIONS

DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOX, doxorubicin; EPR, enhanced permeability and retention; NIRF, near-infrared fluorescence; NOTA, 1,4,7triazacyclononane-1,4,7-triacetic acid); PAMAM, poly-(amidoamine); PBS, phosphate-buffered saline; PET, positron emission tomography; RNAi, RNA interference; saRNA, small activation RNA; siRNA, small interfering RNA; SPECT, single photon emission computed tomography; TEA, triethanolamine; TEM, transmission electron microscope

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