REVIEW ARTICLE

Molecular engineering of dendrimer nanovectors for siRNA delivery and gene silencing

Yu Cao[1], Xiaoxuan Liu[2], Ling Peng (✉)[1]

1 Aix-Marseille Université, CNRS, Centre Interdisciplinaire de Nanoscience de Marseille, UMR 7325, Equipe Labellisée Ligue Contre le Caner, 13288 Marseille, France
2 State Key Laboratory of Natural Medicines and Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, Center of Drug Discovery, Center of Advanced Pharmaceuticals and Biomaterials, China Pharmaceutical University, Nanjing 210009, China

Abstract Small interfering RNA (siRNA) therapeutics hold great promise to treat a variety of diseases, as long as they can be delivered safely and effectively into cells. Dendrimers are appealing vectors for siRNA delivery by virtue of their well-defined molecular architecture and multivalent cooperativity. However, the clinical translation of RNA therapeutics mediated by dendrimer delivery is hampered by the lack of dendrimers that are of high quality to meet good manufacturing practice standard. In this context, we have developed small amphiphilic dendrimers that self-assemble into supramolecular structures, which mimic high-generation dendrimers synthesized with covalent construction, yet are easy to produce in large amount and superior quality. Indeed, the concept of supramolecular dendrimers has proved to be very promising, and has opened up a new avenue for dendrimer-mediated siRNA delivery. A series of self-assembling supramolecular dendrimers have consequently been established, some of them out-performing the currently available nonviral vectors in delivering siRNA to various cell types *in vitro* and *in vivo*, including human primary cells and stem cells. This short review presents a brief introduction to RNAi therapeutics, the obstacles to their delivery and the advantages of dendrimer delivery vectors as well as our bio-inspired structurally flexible dendrimers for siRNA delivery. We then highlight our efforts in creating self-assembling amphiphilic dendrimers to construct supramolecular dendrimer nanosystems for effective siRNA delivery as well as the related structural alterations to enhance delivery efficiency. The advent of self-assembling supramolecular dendrimer nanovectors holds great promise and heralds a new era of dendrimer-mediated delivery of RNA therapeutics in biomedical applications.

1   RNA interference and siRNA therapeutics

RNA therapeutics based on RNA interference (RNAi) provide an enormous opportunity for treating a variety of diseases by virtue of the ability of small interfering RNA (siRNA) to turn off the expression of specific disease-related genes [1–4]. RNAi is a sequence-specific gene silencing mechanism triggered by siRNA (Fig. 1) [5,6]. It was discovered in 1998 by Fire and Mello et al. [5], who were consequently awarded the Nobel Prize in 2006. The natural RNAi process involves the cleavage of endogenous long double-stranded RNAs into short siRNAs (~21–23 nucleotides in length) by the Dicer endonuclease [6]. The resulting siRNA is then incorporated into the multiprotein RNA-induced silencing complex (RISC). The double-stranded siRNA is subsequently unwound, with the sense or "passenger" strand being degraded while the antisense or "guide" strand targets the 3′ end of mRNA transcripts through complementary Watson-Crick base-pairing. The mRNA is then degraded by the endonuclease activity of Argonaute 2 (Ago2), a RISC subunit, preventing translation of the mRNA into protein, and hence resulting in gene silencing (Fig. 1) [7]. Following the degradation of the target mRNA, the activated RISC complex is regenerated and can then mediate further cycles of mRNA binding and cleavage [8].

Synthetic siRNAs can be designed to be complementary to any gene for which the sequence is available, and can therefore be applied to target and down-regulate disease genes for therapeutic purposes [1–4]. By virtue of the catalytic nature of the RNAi mechanism, siRNAs have a stronger and more prolonged therapeutic effect when compared with antisense oligonucleotides and ribozymes.

The potential of siRNA to catalyze the specific down-regulation of any disease gene with known sequence has opened up a new era of RNA therapeutics, which has the potential to revolutionize the treatment of many diseases, including those that are incurable or difficult to treat because of drug resistance, or the absence of effective drugs or druggable targets.

2   Challenges to siRNA delivery

Based on the RNAi mechanism, siRNA molecules targeting specific disease-related genes with known sequences can be easily devised according to Watson-Crick base-pairing and then synthesized readily according to good manufacturing practice (GMP) standards as therapeutic agents. However, naked siRNAs are not suitable for therapeutic administration because they are not stable and can be rapidly degraded by enzymes such as nucleases and esterases that are present in the body. Furthermore, siRNAs are highly anionic hydrophilic molecules with a molecular weight over 12000 Daltons, and therefore cannot readily cross biological membranes to enter into cells. Consequently, there is a high demand for safe and efficient delivery systems, which can protect the siRNA cargo from degradation, shield the high density of negative charges, convey the siRNA to the site of interest, and enter into cells.

Both viral and nonviral vectors have been explored for siRNA delivery, and viral vectors are currently the most effective. However, growing concerns over the safety and immunogenicity of viral vectors have resulted in enormous efforts over the last decade to develop nonviral vectors for siRNA delivery [9–11]. These efforts have given rise to a variety of novel synthetic vectors composed of lipids, polymers, and inorganic materials such as carbon nanotubes, gold nanoparticles and quantum dots. To be effective for siRNA delivery, an ideal delivery vector must fulfil four main criteria. Firstly, it must be able to shelter the siRNA from degradation in the bloodstream. Secondly, it must be able to deliver the siRNA specifically to the target site, in order to avoid unwanted side-effects from nonspecific delivery. Thirdly, the vector must have the ability to promote cell uptake and to escape from endosomes once inside the cell. Finally, the carrier must release the siRNA efficiently so that it can interact with the RISC and activate the RNAi mechanism to effectively silence the target gene (Fig. 2). The major disadvantage of nonviral carriers is that their delivery efficiency is much lower than viral vectors. Therefore, a great endeavour has been directed to develop nonviral carriers with better transfection efficiency.

3   Dendrimers for siRNA delivery

Among the various nonviral vectors, dendrimers, a special class of synthetic polymers characterized by a distinct
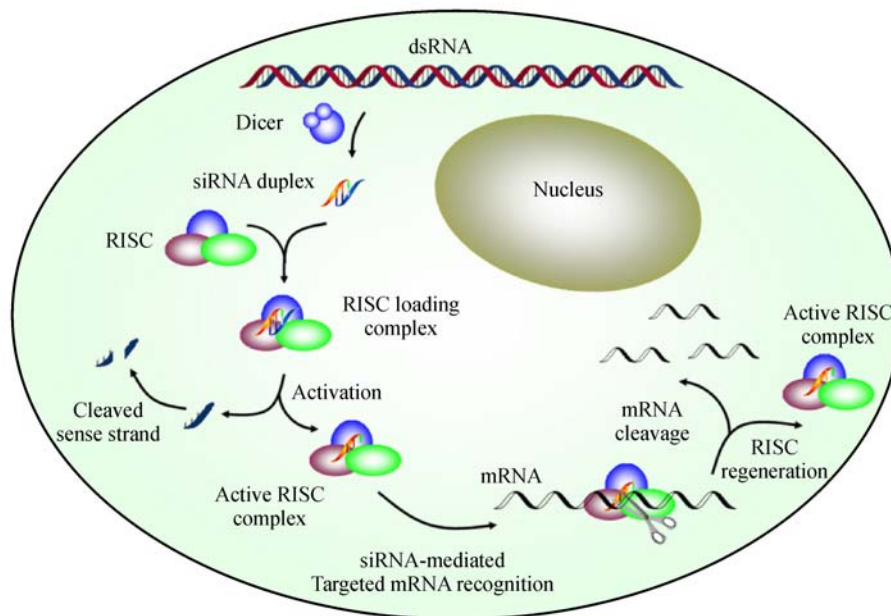

Fig. 1   The mechanism of RNA interference. Silencing is triggered by long double-stranded RNAs, which are recognized and processed into small interfering RNAs (siRNAs) by the enzyme Dicer. The duplex siRNAs are then passed to the RISC (RNA-induced silencing complex), which is activated by unwinding of the siRNA. The sense strand of the siRNA is cleaved and discarded, while the antisense strand remains associated with the active RISC complex, guiding it to a target mRNA containing a complementary sequence. The complex then cleaves the mRNA, resulting in silencing of the targeted gene. The RISC can destroy multiple identical transcripts by undergoing further catalytic cycles of binding and cleavage of target mRNAs
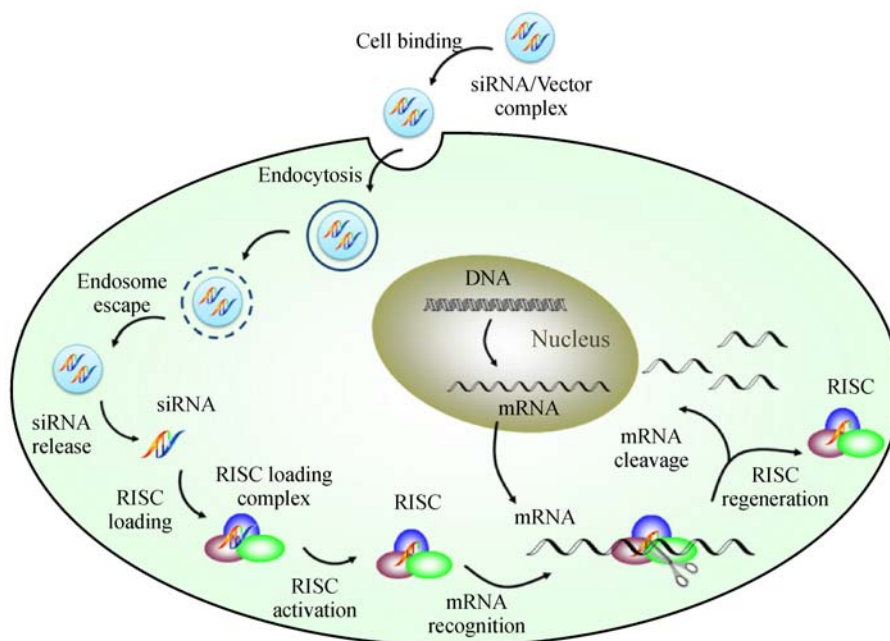
Fig. 2 Nonviral vector-mediated siRNA delivery and gene silencing

ramified molecular architecture and unique multivalence, represent a promising delivery platform for siRNA therapeutics [12,13]. They differ from traditional polymer vectors in that they possess a well-defined structure with three distinct structural domains (Fig. 3): (i) a central core, where dendrimer growth begins; (ii) branching units which allow dendrimer growth in geometrically organized radial layers known as generations (**G**); and (iii) an abundance of terminal functionalities on the surface, which confer specific properties along with multivalent cooperativity on the dendrimer [14]. It is worth mentioning that the central core itself is generation 0 (G_0), while generation 1 (G_1), generation 2 (G_2) and generation 3 (G_3) refer to dendrimers with the first, second and third levels of branching, respectively (Fig. 3). In addition, the structure of a dendrimer can be controlled with precision during the stepwise synthesis process, which can be carried out via either a divergent strategy or a convergent strategy or a combination of both [14,15]. These unique features make dendrimers distinctly different from linear and hyperbranched polymers.

To date, a multitude of dendrimers have been investigated to for their potential in siRNA delivery. Among them, poly(amidoamine) dendrimers, also called PAMAM dendrimers (Fig. 4) have been the most extensively explored. PAMAM dendrimers were initially synthesized as protein mimics by Tomalia and co-workers in 1985 [16], and have since been widely studied as nonviral vectors for DNA delivery [17–21]. These dendrimers terminate with primary amine groups, and are therefore positively charged at physiological pH (7.4). Consequently, PAMAM dendrimers can readily condense polyanionic nucleic acids
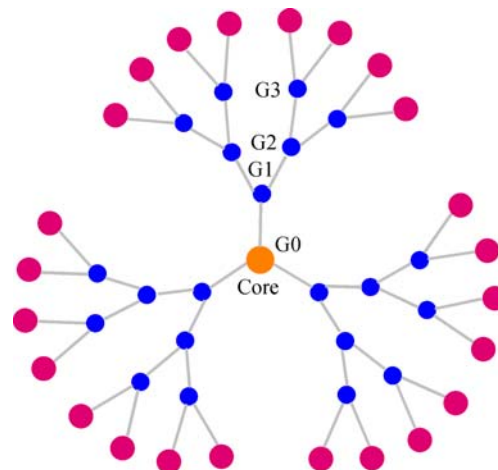

Fig. 3 General presentation of the structure of a dendrimer, which is composed of a central core, the repetitive branching units which form the generations and the terminal groups. The central core itself is generation 0 (G_0); generation 1 (G_1), generation 2 (G_2) and generation 3 (G_3) refer to dendrimers with the first, second and third levels of branching, respectively

into dendriplexes via electrostatic interactions. Another important feature of PAMAM dendrimers is that they contain tertiary amines in their interior structure, which can mediate the "protein sponge" effect in acidic intracellular vesicles (endosomes and lysosomes). Protonation of tertiary amines is thought to lead to osmotic imbalance, causing rupture of the endosomal vesicles, disassembly of the dendriplexes, and release of the nucleic acid cargo into the cytoplasm [22]. Following release, the nucleic acid
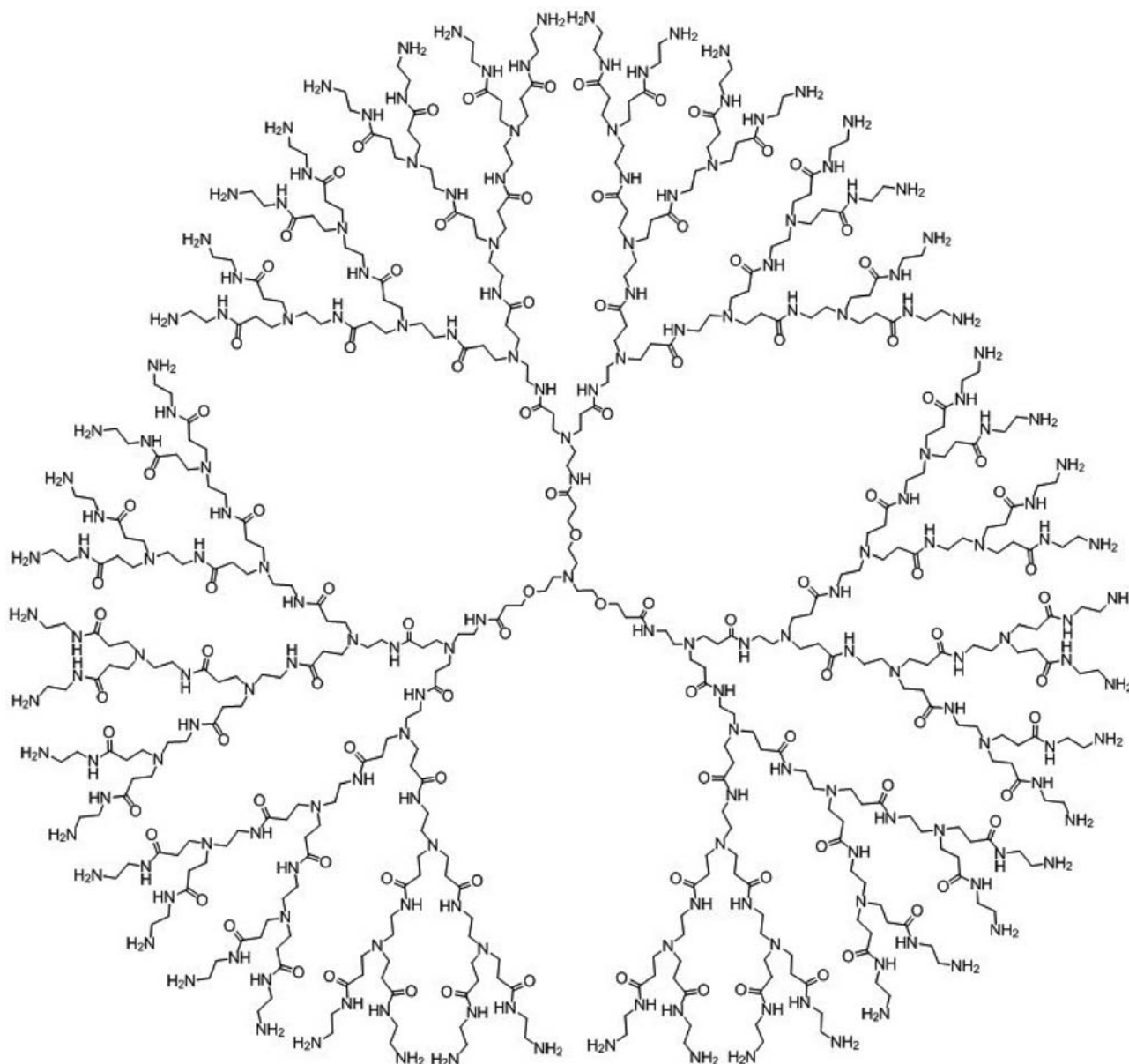
Fig. 4 Molecular structure of a PAMAM dendrimer with a triethanolamine (TEA) core. For clarity, only a generation 4 dendrimer is presented

molecules will eventually enter into the nucleus for DNA transfection or engage with the RNAi machinery to activate the RISC for gene silencing.

Although PAMAM dendrimers have been widely studied as nonviral vectors for DNA delivery [17–21], in the past decade they have also been investigated as siRNA carriers [12,23,24]. In 2005, Juliano et al. tested the siRNA delivery properties of a commercially available PAMAM dendrimer with an ethylenediamine core [25]. Neither the dendrimer itself nor its conjugates were effective for siRNA delivery and gene silencing. Soon afterwards, in 2006, we developed structurally flexible PAMAM dendrimers and used them achieve effective siRNA delivery

and successful gene silencing [26]. These structurally flexible dendrimers [27] were conceived by inspiration from the histone, which has dynamic structure via conformational change when binds to DNA for post translational modification while dissemble DNA to prevent transcription [28]. The devised dendrimers have an extended TEA core (Fig. 4) [27], which endows the dendrimers with more internal space to accommodate the branching units, thereby imparting structural flexibility to the resulting dendrimers [29]. In other words, the branching units in TEA-core-based dendrimers are less densely packed, which means that the dendrimers are more accessible to water and nucleic acids and thus have

enhanced nucleic acid binding and release properties [29–31]. Indeed, our structurally flexible TEA-core dendrimers are able to bind siRNA to form stable and uniform nanoparticles with a size of ~70 nm [32], which can effectively protect the siRNA from degradation and facilitate cellular uptake of siRNA via endocytosis, preferentially macropinocytosis (Fig. 5) [30]. After entering a cell, the siRNA molecules can escape from the endosome through swelling and breakage of the siRNA/dendrimer complexes, which is promoted by the "proton sponge" effect as described above. Consequently, these PAMAM dendrimers make excellent vectors for delivering RNA therapeutics in various disease models such as prostate cancer [30,31,33–35], liver cancer [36], ovarian cancer [37], glioblastoma [38,39] and HIV infection [40], etc. One of our dendrimers, the generation 5 of TEA-core PAMAM dendrimer, was scheduled for clinical trial for cancer therapy [23]. Unfortunately, due to the lack of GMP dendrimer, the clinical trial was delayed and finally modified with the use of Smarticles® to replace the dendrimer for delivery of the RNA therapeutics (https://clinicaltrials.gov/ct2/show/NCT02716012).
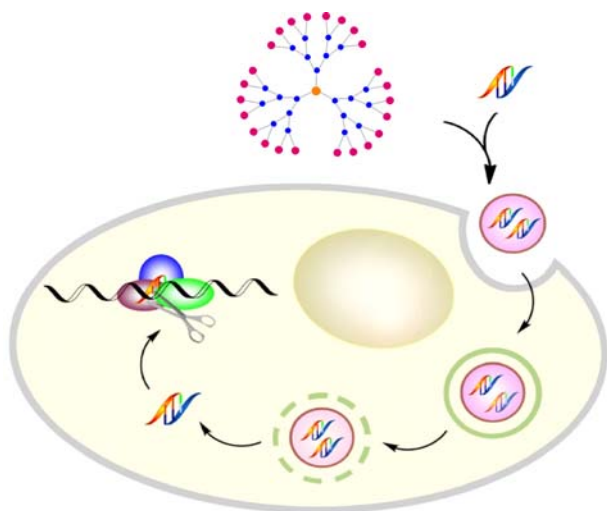

Fig. 5 Cartoon presentation of siRNA delivery mediated by a dendrimer

It should be mentioned that dendrimer synthesis is by no means trivial, but is instead rather tedious, time-consuming and laborious. Different from conventional polymers, which are created by one-pot synthesis, the construction of dendrimers is achieved via stepwise synthesis, similar to that of small molecules. However, unlike small molecules, dendrimer purification is difficult and complicated due to the presence of highly similar side-products [41]. This situation gets even worse when preparing higher generation dendrimers. All these issues mean that the large-scale production of GMP-standard high-generation dendrimers

is technically challenging, which impedes the clinical translation of dendrimer-based therapeutic interventions [41].

4 Self-assembling supramolecular dendrimer vectors

Since GMP production of high-generation PAMAM dendrimers is challenging, it is desirable and preferable to explore small, easy-to-synthesize low-generation dendrimers for siRNA delivery. We then came up with the idea of using small amphiphilic dendrimers [42] to create large supramolecular dendrimers through self-assembly (Fig. 6 (A)) [43], in the hope that these non-covalently-created supramolecular dendrimers could mimic the covalently-constructed high-generation dendrimers in size, structure and function — in particular, for siRNA delivery.

With this in mind, we developed an amphiphilic dendrimer (**1**) bearing a hydrophobic C18 alkyl chain and a hydrophilic PAMAM dendron with 8 amine terminals (Fig. 6(B)) [42]. This dendrimer self-assembles into nanoparticles with a size of ~7.0 nm [43], which is in the range of typical nanomicelles. Remarkably, the dimensions of these self-assembled nanomicelles are similar to those of high-generation structurally flexible TEA-core dendrimers [29]. This suggests that the nanostructures generated by self-assembly of low-generation dendrimers are structural mimics of traditional covalently synthesized high-generation dendrimers (Fig. 6(A)). Most importantly, this supramolecular dendrimer mimic has been shown to deliver siRNA effectively to various cell types, leading to potent gene silencing *in vitro* and *in vivo* [42,43].

It is noteworthy that small PAMAM dendrimers either without a hydrophobic chain or with a hydrophilic PEG chain (**2** and **3** in Fig. 7) are devoid of amphiphilic character and cannot form micellar supramolecular dendrimers for siRNA delivery [43]. These results can be ascribed to the intrinsic incapacity of these dendrimers to self-assemble into robust supramolecular structures that mimic high-generation dendrimers. Thus, the presence of the positively-charged dendron structure by itself is insufficient for effective siRNA delivery.

Furthermore, amphiphilic dendrimers with either lower generation dendrons [42] (**4** and **5** in Fig. 7) or shorter alkyl chains [43] (**6** and **7** in Fig. 7) also failed to generate notable gene silencing. This highlights that both the size of the PAMAM dendron and the length of the alkyl chain have an important effect on the delivery efficiency [43,44]. If the generation number is too low (as with **4** and **5**), the multivalency will be small and the dendrimers will not be able to form a strong electrostatic interaction with siRNA molecules. If the alkyl chain length is too short (as with **6** and **7**), the ability to form hydrophobic interactions will be
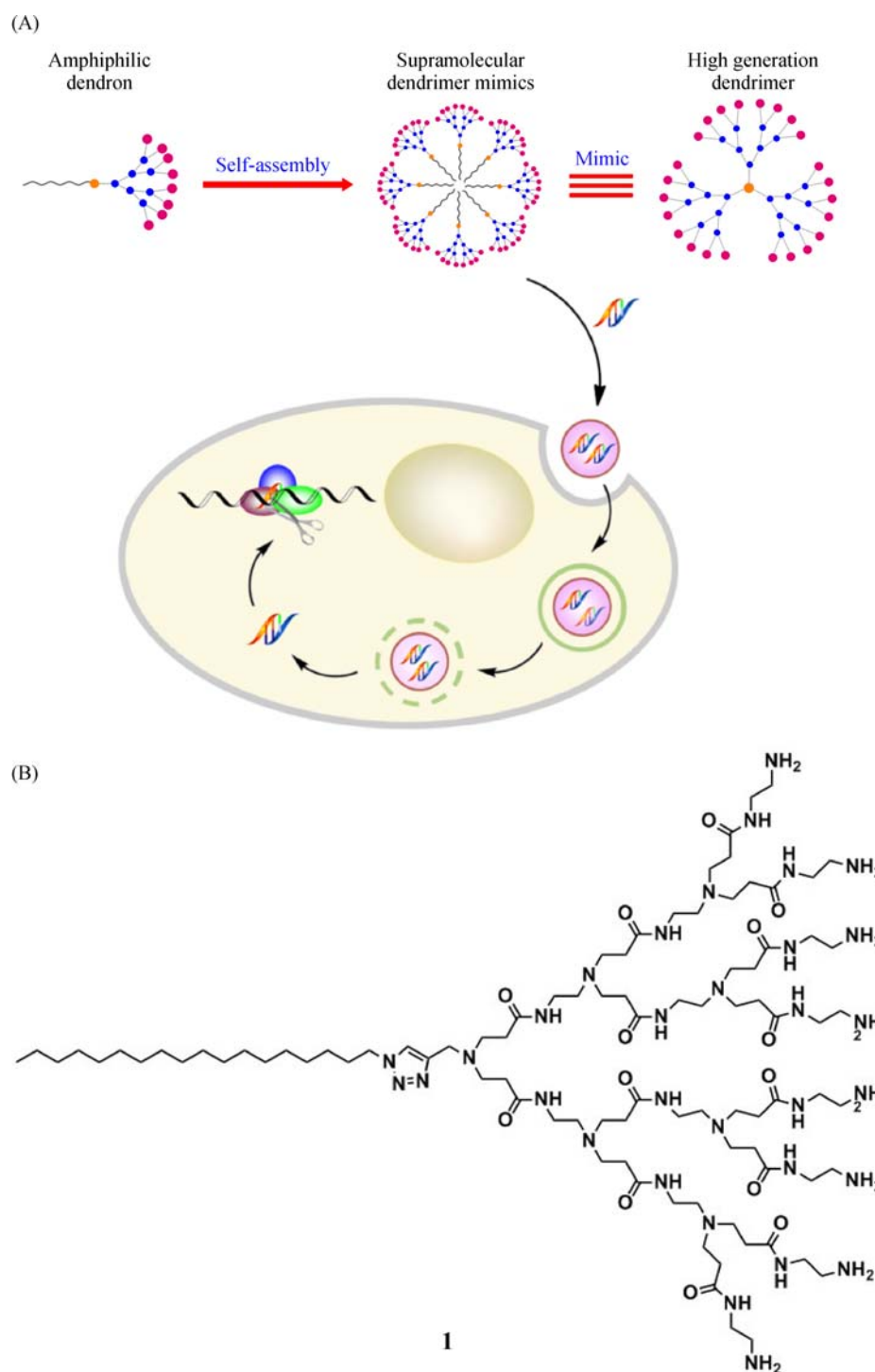
Fig. 6 (A) Self-assembly of a small amphiphilic dendrimer to create a supramolecular dendrimer that mimics a covalently synthesized high-generation dendrimer for siRNA delivery [43]; (B) amphiphilic dendrimer **1**, carrying a hydrophobic C18 alkyl chain and a hydrophilic PAMAM dendron with 8 terminal amine groups [42]

weak and the molecules will not be able to self-assemble into stable supramolecular dendrimers for effective siRNA delivery.

Interestingly, PAMAM dendrimers containing longer hydrophobic tails (**8** and **9** in Fig. 7) were less effective for siRNA delivery, although both **8** and **9** are able to form more stable and more robust nanomicellar supramolecular dendrimers than **1** [43]. This is because the stable supramolecular dendrimers generated with **8** or **9** form complexes with siRNA which are too compact and stable to disassemble, thus impeding intracellular siRNA release and thereby reducing the efficiency of gene silencing.
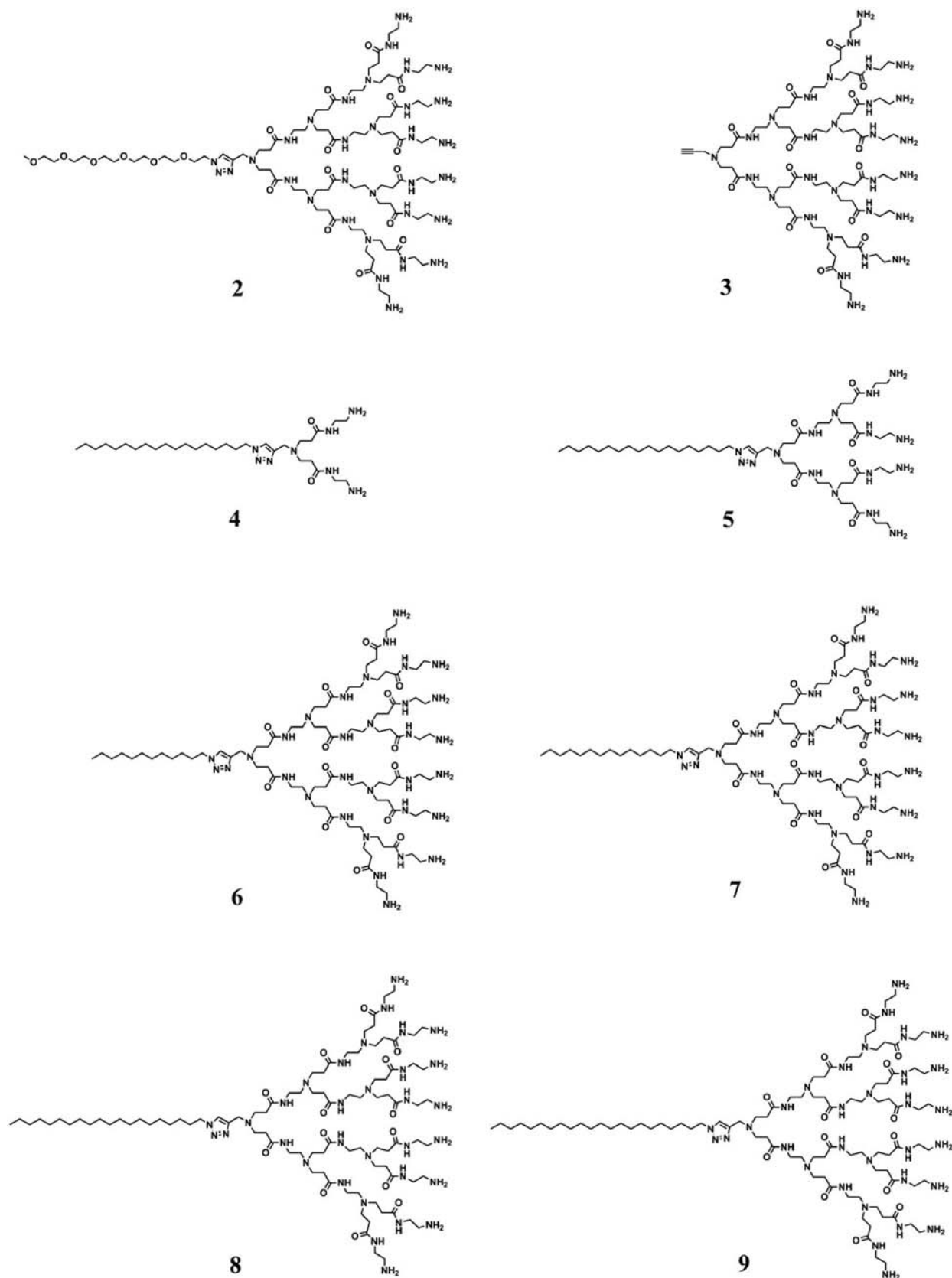
Fig. 7 Structural derivatives of the amphiphilic dendrimer **1** [42,43]

Therefore, it is important to balance the length of the hydrophobic chain and the generation number of the hydrophilic dendritic entity in order to ensure the ideal properties for self-assembly into supramolecular nanostructures to achieve effective gene silencing. The self-assembled supramolecular dendrimers formed by **1** possess the best possible siRNA binding strength alongside the best possible capacity for releasing siRNA. These combined properties allow the resulting supramolecular dendrimer to transport the siRNA through the circulatory system, then enter the target cell and deliver the siRNA cargo to the RNAi machinery for siRNA-mediated gene silencing.

In order to further enhance the delivery efficiency, we conjugated **1** with arginine to create an arginine-terminated dendrimer **10** (Fig. 8), with the aim of capitalizing on the cell-penetrating advantages of arginine-rich motifs for siRNA delivery [45]. It is well known that cell-penetrating peptides bearing arginine-rich motifs can favorably promote cellular uptake [46]. This is because under physiological conditions (pH 7.4), each arginine residue harbors a positively-charged primary amine group and a positively-charged guanidinium moiety. These two groups can interact with negatively-charged components of the plasma membrane, including proteoglycans and the phosphate groups in lipids, to facilitate membrane penetration. In addition, arginine residues can interact with cell membranes through divalent H-bonding of the guanidinium moiety with the phosphate/carboxylate/sulfate groups on the cell surface. We hence reasoned that adding arginine residues to the termini of our dendrimer might also improve cell penetration and cellular uptake of the siRNA/dendrimer complexes. Indeed, compared to the corresponding non-arginine counterpart **1**, dendrimer **10** shows significantly enhanced cell uptake of siRNA, leading to more efficient siRNA delivery and better gene silencing in different human cell types such as the prostate cancer cell line PC-3 and hematopoietic CD34$^+$ stem cells [45]. This work highlights that addition of arginine residues to the amphiphilic dendrimer surface is a useful strategy to increase the efficiency of siRNA delivery.

In another strategy to improve siRNA delivery, we modified the hydrophobic entity of **1** by appending two hydrophobic C18 alkyl chains, leading to the amphiphilic dendrimer **11** (Fig. 9(A)) [47]. Interestingly, this dendrimer spontaneously self-assembled into vesicle-like dendrimersome nanostructures [48] (Fig. 9(B)) instead of the micellar structures as formed by **1**. This finding is
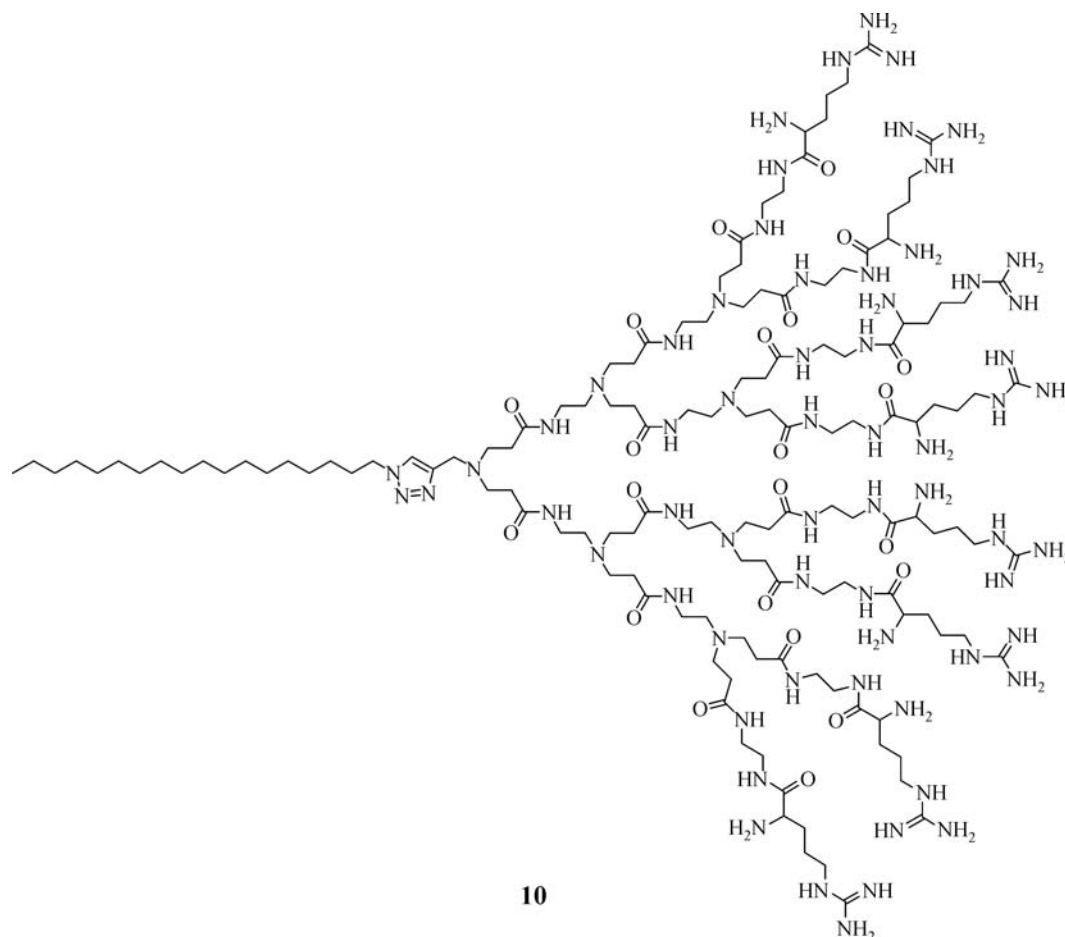

Fig. 8 Arginine-terminated amphiphilic dendrimer **10** to enhance cellular uptake for more efficient siRNA delivery [45]

somewhat reminiscent of lipids and detergents, with lipids often generating vesicular structures while detergents preferentially form micelles. Most importantly, the vesicle-like dendrimersomes formed by **11** are able to undergo concurrent structural rearrangement into small, spherical micelles upon interaction with siRNA (Fig. 9(B)). This vesicular-to-micellar structural transition will allow **11** to maximally expose its positively-charged terminal groups and form stronger electrostatic interactions with the anionic phosphate groups of siRNA. This will effectively entrap and condense siRNA into stable nanoparticles in order to shelter siRNA from degradation and deliver it to the desired site. In addition, the dendrimersomes formed by **11** are able to harness the delivery advantages of both lipid and dendrimer vectors, and are largely better than other existing nonviral vectors for delivering siRNA to a
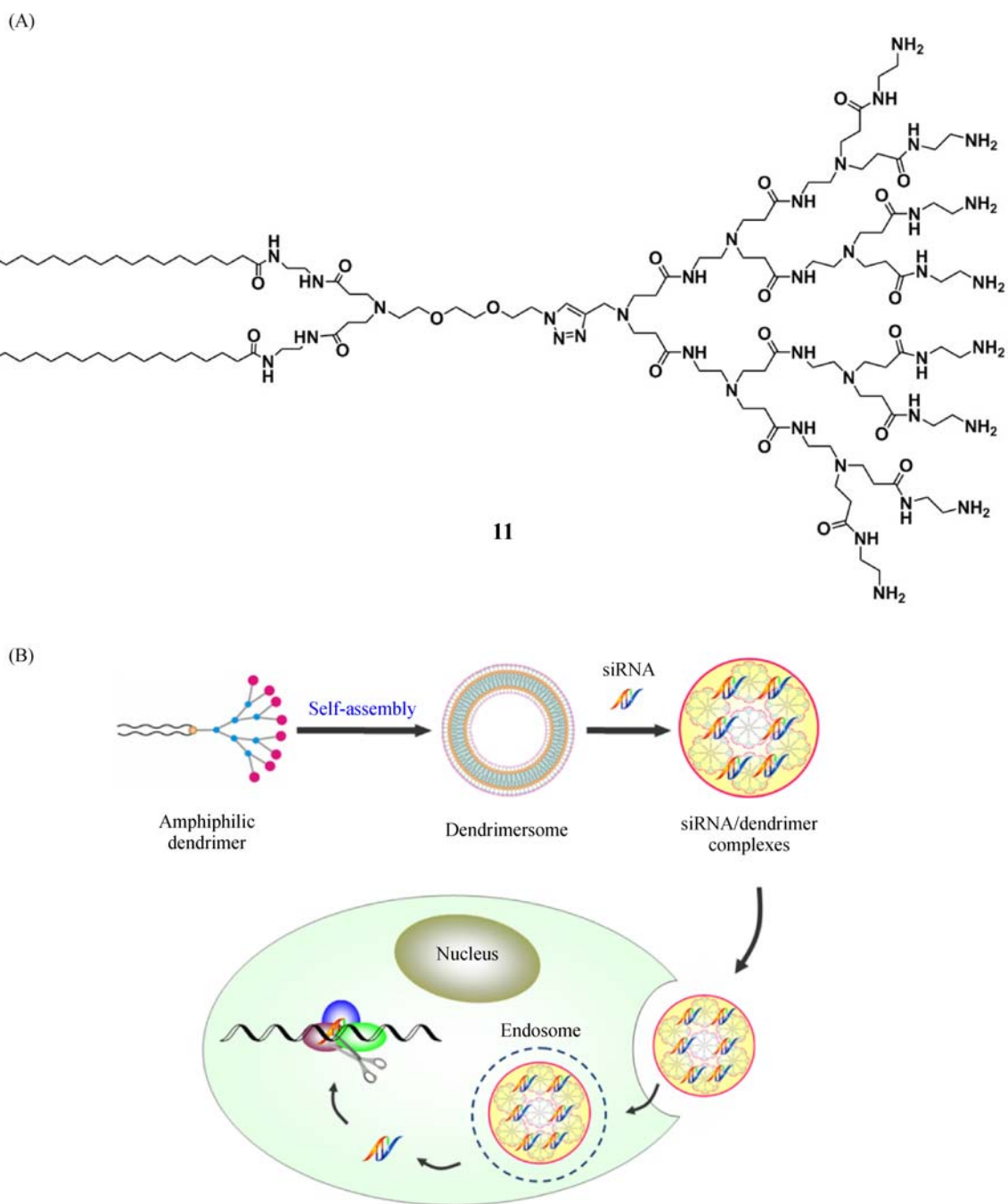
(A)

11

(B)

Fig. 9 (A) Amphiphilic dendrimer **11** bearing two hydrophobic alkyl chains and a hydrophilic PAMAM dendron; (B) **11** forms dendrimersomes, which upon addition of siRNA, undergo spontaneous rearrangement into micelles, in order to provide maximal exposure of the positively-charged dendrimer terminals for interaction with the negatively-charged siRNA [47]

variety of different cell types *in vitro* and *in vivo* [47]. This system is also effective for targeting human primary cells and stem cells, which are highly challenging cell types for siRNA delivery. Thus, **11** has the unique ability to undergo dynamic self-assembly and rearrangement in the presence of siRNA to form adaptive supramolecular assemblies which combine the advantageous features of both lipid- and polymer-based vectors. These properties of **11**, together with the fact that it is easy to synthesize, hold great promise for highly effective delivery of siRNA to a wide variety of cell types.

In our further effort to develop novel amphiphilic dendrimers for specific siRNA delivery, we have established a fluorinated bola-amphiphilic dendrimer for on-demand delivery of siRNA in response to the high level of reactive oxygen species (ROS) in cancer cells (Fig. 10) [49]. ROS are constantly generated within cancer cells because of the highly stressful environment caused by rapid and uncontrollable cancer cell proliferation [50,51]. We capitalized on this characteristic feature of cancer cells to develop ROS-triggered specific delivery of siRNA. The bola-amphiphilic dendrimer **12** features a bola-lipid/dendrimer hybrid harboring a ROS-sensitive thioacetal functionality at the central core and two hydrophilic PAMAM dendrons at the terminals. The hydrophobic "bola-lipid" core scaffold is designed to mimic the robust and strong assembly properties of bola-amphiphiles observed in extremophile archaebacteria [52], whereas the two PAMAM dendrons interact with the siRNA and compact it into nanoparticles to protect it and promote its
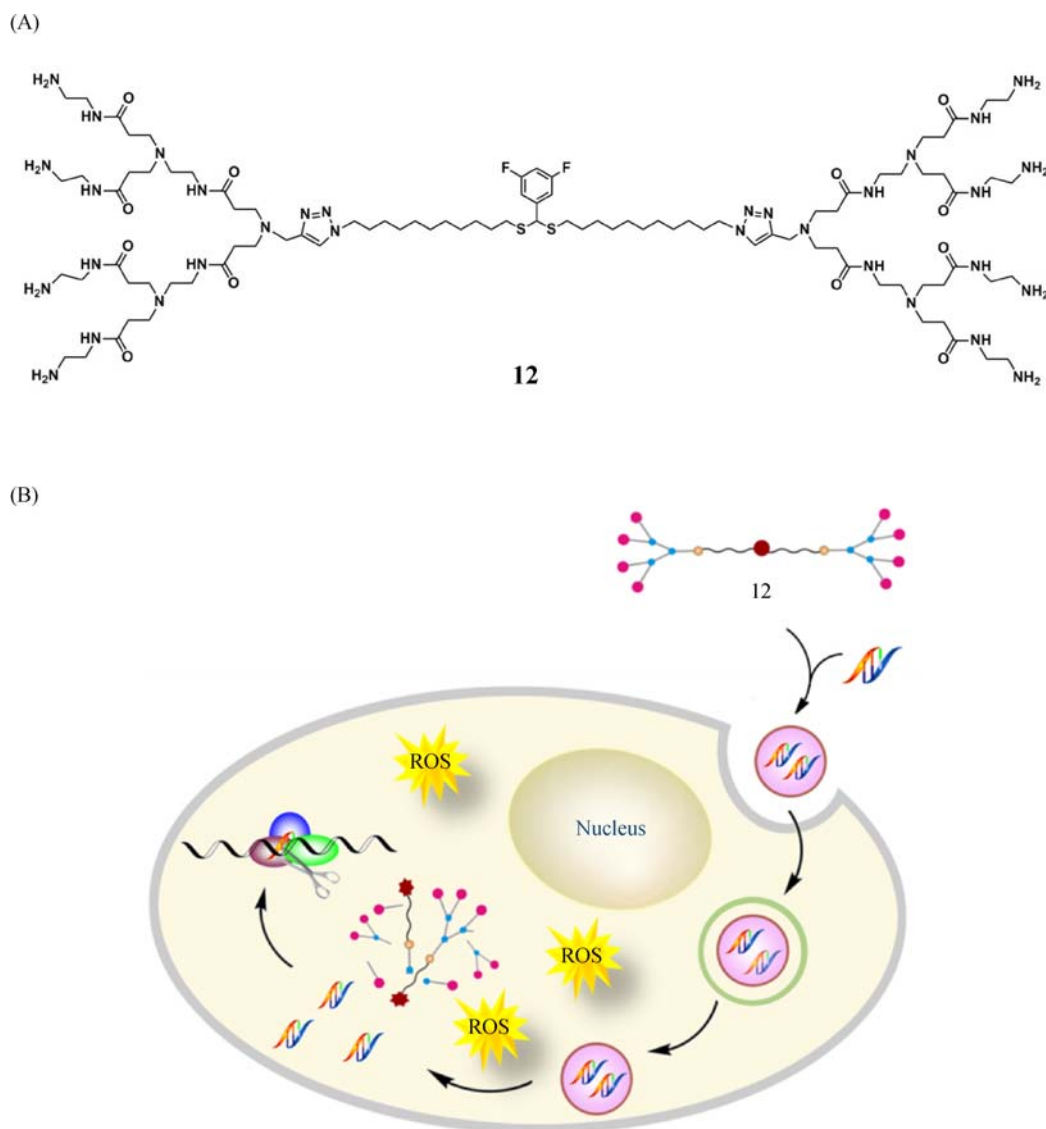

Fig. 10 The bola-amphiphilic dendrimer **12** designed for ROS-responsive siRNA delivery. (**A**) Chemical structure of **12**; (**B**) schematic representation of **12** for the ROS-triggered delivery of siRNA and consequential gene silencing. The dendrimer **12** is able to form nanosized complexes with siRNA, which can be internalized by the cancer cell before releasing siRNA in response to ROS, leading to effective gene silencing [49]

cellular uptake. In the presence of ROS, the thioacetal entity in the bola core is decomposed effectively, concurrently promoting siRNA unpacking for siRNA delivery and potent gene silencing in ROS-abundant cancer cells [49]. In addition, the hydrophobic bola-core in **12** is shorter than the native phospholipid bilayer, which can prevent the insertion of **12** into the cell membrane, thus obviating deleterious effects on cell membrane integrity and cell viability. Indeed, **12** is devoid of serum hemolysis and cytotoxic effects. This work represents the first report on a bola-amphiphilic dendrimer for on-demand siRNA delivery in response to ROS in cancer cells. Also of note is the presence of fluorine tags within this dendrimer, allowing the tracking of the ROS-responsive delivery process using ^{19}F high-resolution magic angle spinning nuclear magnetic resonance (HRMAS NMR) [49]. ^{19}F HRMAS NMR is a sensitive, non-invasive technique for analyzing ^{19}F-containing molecules in intact cells, and is ideal for tracking fluorinated **12** because there are no endogenous intracellular fluorinated compounds, which means that the background ^{19}F-NMR signal is minimized [53]. This study also opens a new perspective on the development of fluorinated dendrimers for ^{19}F-NMR-based cancer detection along with dendrimer-mediated siRNA therapeutic intervention in cancer treatment.

5 Conclusions and prospects

Dendrimers are considered as promising delivery platforms for diverse therapeutic agents because of their distinct molecular architecture, precisely controlled structure and unique multivalence, which is characterized by uniformly-spaced radiating branching units in the interior and multiple terminal groups on the surface. During the last 10 years, dendrimers have emerged as effective nonviral vectors for siRNA delivery. In particular, we have established bio-inspired structurally flexible TEA-core PAMAM dendrimers as excellent vectors for siRNA delivery in various disease models *in vitro* and *in vivo*. One of our TEA-core PAMAM dendrimers, namely the generation 5 dendrimer, was scheduled for clinical trial to deliver RNA therapeutics for cancer treatment. Unfortunately, due to the shortage of GMP-standard PAMAM dendrimer, the trial was delayed and finally modified by replacing the dendrimer vector with Smarticles^R as the delivery agent.

To overcome this problem, we have introduced the concept of using small amphiphilic dendrimers to generate self-assembling supramolecular dendrimers, which can mimic covalently-constructed high-generation dendrimers for siRNA delivery. A series of amphiphilic dendrimers have been therefore established and their self-assembling features to generate supramolecular dendrimers for siRNA delivery have been studied. Collectively, our studies have revealed that an optimal balance between the hydrophobic component and the hydrophilic entity is crucial for self-assembly into stable and robust supramolecular dendrimers which can mimic structurally well-defined covalent high-generation dendrimers. These supramolecular assemblies can bind siRNA, shelter it from degradation in the circulation, and release it once internalized within the cell for effective RNA interference and gene silencing. The convenient synthesis of these small amphiphilic dendrimers via click chemistry allows us to carefully devise and gauge building blocks with an ideal hydrophobic/hydrophilic balance in order to identify the best candidates for siRNA delivery. Also, the easy protocol for synthesis of small amphiphilic dendrimers along with the simple purification process will greatly facilitate their eventual GMP production and accelerate their translation into real therapeutic interventions. Therefore, the advent of self-assembling supramolecular dendrimer nanostructures holds great promise and heralds a new era of dendrimer-mediated delivery of RNA therapeutics for biomedical applications.

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Dr. Ling Peng is currently a research director at the French National Scientific Research Center (CNRS) and a group leader at the Interdisciplinary Center of Nanoscience in Marseille (CINaM), France. She carried out her undergraduate study in polymer science with Prof. Chen Rongshi at Nanjing University in China, her Ph.D. program in organic chemistry with Prof. Albert Eschenmoser at the Swiss Federal Institute of Technology in Zurich, Switzerland, and her postdoctoral research in pharmaceutical science with Prof. Maurice Goeldner at the Louis Pasteur University of Strasbourg in France. She was recruited as a research scientist at CNRS in 1997, and promoted to CNRS research director in 2008. She is working actively at the interface of chemistry and biology, and mainly focused on developing functional dendrimers for drug delivery, and molecular probes for understanding and exploring biological events for drug discovery. In particular, she has pioneered bio-inspired structurally flexible dendrimers for nucleic acid delivery in gene therapy, and inaugurated the concept of self-assembling supramolecular dendrimers for drug delivery. Dr. Ling Peng is currently a member of the Editorial Advisory Board of *Molecular Pharmaceutics,* section editor of drug delivery for *Current Medicinal Chemistry* and Assistant Editor of *New Journal of Chemistry.*