Self-assembly of amphiphilic Janus dendrimers into uniform onion-like dendrimersomes with predictable size and number of bilayers

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A constitutional isomeric library synthesized by a modular approach has been used to discover six amphiphilic Janus dendrimer primary structures, which self-assemble into uniform onion-like vesicles with predictable dimensions and number of internal bilayers. These vesicles, denoted onion-like dendrimersomes, are assembled by simple injection of a solution of Janus dendrimer in a water-miscible solvent into water or buffer. These dendrimersomes provide mimics of double-bilayer and multibilayer biological membranes with dimensions and number of bilayers predicted by the Janus compound concentration in water. The simple injection method of preparation is accessible without any special equipment, generating uniform vesicles, and thus provides a promising tool for fundamental studies as well as technological applications in nanomedicine and other fields.

synthetic membranes | biomembrane mimics | multibilayer vesicles

Most living organisms contain single-bilayer membranes composed of lipids, glycolipids, cholesterol, transmembrane proteins, and glycoproteins (1). Gram-negative bacteria (2, 3) and the cell nucleus (4), however, exhibit a strikingly special envelope that consists of a concentric double-bilayer membrane. More complex membranes are also encountered in cells and their various organelles, such as multivesicular structures of eukaryotic cells (5) and endosomes (6), and multibilayer structures of endoplasmic reticulum (7, 8), myelin (9, 10), and multilamellar bodies (11, 12). This diversity of biological membranes inspired corresponding biological mimics. Liposomes (Fig. 1) self-assembled from phospholipids are the first mimics of single-bilayer biological membranes (13–16), but they are polydisperse, unstable, and permeable (14). Stealth liposomes coassembled from phospholipids, cholesterol, and phospholipids conjugated with poly(ethylene glycol) exhibit improved stability, permeability, and mechanical properties (17–20). Polymersomes (21–24) assembled from amphiphilic block copolymers exhibit better mechanical properties and permeability, but are not always biocompatible and are polydisperse. Dendrimersomes (25–28) self-assembled from amphiphilic Janus dendrimers and minidendrimers (26–28) have also been elaborated to mimic single-bilayer biological membranes. Amphiphilic Janus dendrimers take advantage of multivalency both in their hydrophobic and hydrophilic parts (23, 29–32). Dendrimersomes are assembled by simple injection (33) of a solution of an amphiphilic Janus dendrimer (26) in a water-soluble solvent into water or buffer and produce uniform (34), impermeable, and stable vesicles with excellent mechanical properties. In addition, their size and properties can be predicted by their primary structure (27). Amphiphilic Janus glycodendrimers self-assemble into glycodendrimersomes that mimic the glycan ligands of biological membranes (35). They have been demonstrated to be bioactive toward biomedically relevant bacterial, plant, and human lectins, and could have numerous applications in nanomedicine (20).

More complex and functional cell mimics such as multivesicular vesicles (36, 37) and multibilayer onion-like vesicles (38–40) have also been discovered. Multivesicular vesicles compartmentalize a larger vesicle (37) whereas multibilayer onion-like vesicles consist of concentric alternating bilayers (40). Currently multibilayer vesicles are obtained by very complex and time-consuming methods that do not control their size (39) and size distribution (40) in a precise way. Here we report the discovery of “single-single” (28) amphiphilic Janus dendrimer primary structures that self-assemble into uniform multibilayer onion-like dendrimersomes (Fig. 1) with predictable size and number of bilayers by simple injection of their solution into water or buffer.

Results and Discussion

A modular synthetic approach was used to prepare a library consisting of eight constitutional isomeric amide-containing single-single (28) amphiphilic Janus dendrimers (26) (Fig. 2). “Single-single” amphiphilic Janus dendrimer refers to a compound constructed from a “single” hydrophilic and a “single” hydrophobic dendron (28), rather than from “twin” hydrophilic and “twin” hydrophobic dendrons (26, 27). This modular synthetic approach involves coupling of the hydrophilic acids **3b** and **5a–e** with the hydrophobic amine minidendron (41) **8a**, or hydrophobic acid minidendron **3a** with the hydrophilic amine **8b**. Dodecyl groups and triethylene glycol fragments were used for hydrophobicity and hydrophilicity, respectively. The structures,

Significance

Simple injection of a solution of amphiphilic Janus dendrimer with specific primary structure into water or buffer has been shown to yield uniform submicrometer-size onion-like vesicles denoted dendrimersomes. The size and number of alternating internally confined bilayers is predicted by the final concentration of the Janus dendrimer. Onion-like dendrimersomes provide mimics of various biological membranes and can be elaborated to provide time-dependent delivery of drugs. Their ease of preparation contrasts with conventional methods used to make onion-like vesicles that are both complicated and time-consuming.

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Fig. 1. Strategies for the preparation of single-bilayer vesicles and multibilayer onion-like vesicles.

and short notations of these single–single (28) amphiphilic Janus dendrimers are summarized in Fig. 2. This report will demonstrate the synthetic capabilities of first-generation dendrons, denoted previously as minidendrons (41), as models or maquettes for the discovery of novel architectural motifs that may be accessible also from higher generations of Janus dendrimers during self-assembly in water. The role of these minidendrons (41–43) and Janus minidendrimers is analogous to that of simple peptides used in the understanding of molecular engineering involved during the assembly of more complex proteins, or of maquettes used by sculptors and architects to appreciate various aspects of full-size objects (41, 44). The minidendron concept has been already demonstrated to be successful for the discovery of a variety of novel complex architectures and functions (41, 43), the most recent example being in the discovery of supramolecular homochirality by chiral self-sorting during supramolecular helical organization (42).

A tetrahydrofuran (THF) solution of each single–single amphiphilic Janus compound from Fig. 2 (100 μ L) was injected into 2 mL of Millipore water. This method is referred to as direct injection. The size and size distribution of the resulting assemblies analyzed by dynamic light scattering (DLS) and the structures determined by cryogenic-transmission electron microscopy (cryo-TEM) are summarized in Fig. 2. All assemblies exhibited polydispersity (PDI) between 0.12 and 0.18 (Fig. 2 and *SI Appendix*, Tables S1 and S4), which are considered monodisperse in the field of vesicles and liposomes (34).

The primary structures of amphiphilic Janus compounds that self-assemble into onion-like dendrimeresomes were discovered by screening this constitutional isomeric library. Two pairs of constitutional isomers, i.e., (3,5)12G1-CH₂-NH-(3,4,5)-3EO-G1-(OCH₃)₃, **9a** vs (3,5)12G1-NH-CH₂-(3,4,5)-3EO-G1-(OCH₃)₃, **9b** and (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a** vs (3,5)12G1-L-Ala-CH₂-(3,4,5)-3EO-G1-(OCH₃)₃, **10b** were compared to study the constitutional isomeric effect on their self-assembled structure. The two Janus compounds **9a**, **10a** with (3,5)12G1-CH₂- pattern self-assembled into onion-like dendrimeresomes (Fig. 3 *A* and *B*). Based on 400 measurements of cryo-TEM images of onion-like vesicles self-assembled from **9a**, it was found that the spacing between their bilayers was identical, 10.0 ± 0.7 nm (Fig. 4 and *SI Appendix*, Fig. S2). The same feature is valid for the **10a** (*SI Appendix*, Fig. S3). This observation indicates that the number of bilayers of the onion-like dendrimeresomes is proportional to their diameter. On the other hand, the other two constitutional isomers **9b**, **10b** with -CH₂-(3,4,5)-3EO-G1-(OCH₃)₃- pattern self-assembled, respectively, into single-bilayer and onion-like vesicles with different spacing between adjacent bilayers (*SI Appendix*, Fig. S4). More investigations are required to understand this constitutional isomeric effect. Nevertheless, four more amino acid-containing amphiphilic Janus dendrimers **11a**, **12a**, **13a**, and **14a** were designed with (3,5)12G1-CH₂-, which self-assembled into regular onion-like vesicles (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S1). Only **14a** formed a mixture of single-bilayer and onion-like vesicles. The onion-like dendrimeresomes self-assembled from **12a** also exhibit

Fig. 2. Modular synthesis of amphiphilic Janus dendrimers. Reagents and conditions: (i) RCH(NH₂)COOCH₃, HCl, 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), N-methylmorpholine (NMM), THF, 23 °C, 6–8 h; (ii and iv) KOH, EtOH:H₂O, reflux, 1–4 h; (iii and v) CDMT, NMM, THF, 23 °C, 8 h. The diameter (D, in nm) and PDI of the vesicles were measured by DLS (0.5 mg/mL in water solution). The indicated structures in water were determined by cryo-TEM.

Fig. 3. Representative cryo-TEM images of onion-like vesicles self-assembled by injection of THF solution of (A) (3,5)12G1-CH₂-NH-(3,4,5)-3EO-G1-(OCH₃)₃, **9a**; (B) (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a**; (C) (3,5)12G1-CH₂-Gly-(3,4,5)-3EO-G1-(OCH₃)₃, **11a**; and (D) (3,5)12G1-CH₂-L-Ile-(3,4,5)-3EO-G1-(OCH₃)₃, **12a** in water (1 mg/mL). Diameter (D, in nm) and PDI of the onion-like vesicles were measured by DLS.

uniform spacing between the bilayers, 10.3 ± 0.7 nm (Fig. 4). When the amide group from the structure of the Janus compound was replaced with an ester group (28), only single-bilayer vesicles were obtained. This indicates that H bonding is a significant parameter of the primary structures forming onion-like dendrimersomes.

As previously reported for single-bilayer dendrimersomes, their size increases with the increase of the concentration of the Janus dendrimer injected in water (27, 35). Onion-like vesicles were found to exhibit a similar size-concentration dependence. This dependence is exemplified with the onion-like structures formed from (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a**. Their diameter increased from 63 to 289 nm with their concentration ranging from 0.025 to 2.5 mg/mL, as illustrated by the cryo-TEM images with indicated concentrations from Fig. 5. An inspection of Fig. 5 and *SI Appendix, Figs. S1–S5 and S9* provides information on the thickness of the vitrified ice layer from holes of the carbon substrate of the TEM grid. When a darker central part is observed, as in the case of the 12-bilayer vesicle from Fig. 5E, it indicates that the vesicle is protruding out of the vitrified film (45). The vitrified ice is thinner at the center of the hole and thicker near the edges of the hole as sorted by the solvent surface tension (45). This provides a self-sorting of larger vesicles to the edge of the hole and of smaller to the center, and explains why smaller vesicles from the center of the hole have a darker center whereas larger vesicles from the edge are perfectly transparent (Fig. 5). Experimental data of the film thickness measurements reported in the literature based on closely related sample preparations show that the film thickness is ~ 100 nm at the center and up to ~ 430 nm near the edge (46). Judging from the observation of darker or transparent central part of the onion-like vesicles, we estimated the thickness of the vitrified ice films to be ~ 100 nm at the center and ~ 350 nm near the edge of the hole. These values agree with literature data (46). The onion-like vesicles showed narrow size distribution from 0.10 to 0.22 at concentrations ranging from 0.025 to 1.5 mg/mL, which are considered monodisperse (34), whereas the PDI became relatively higher (0.31–0.38) at concentrations from 1.75 to 2.0 mg/mL (Fig. 6B and *SI Appendix, Table S2*). Interestingly, it was observed that the number of bilayers also increased with the final concentration of the Janus compound. At very low concentration (0.025 mg/mL) onion-like dendrimersomes exhibited only two bilayers (Fig. 5A). Therefore, their structure provides a simple mimic of the double-lamellar membrane of Gram-negative bacteria (2, 3) that survive in a more dilute environment than other biological membranes (47). The number of bilayers increased gradually to 4 at 0.1 mg/mL (Fig. 5B), 6 at 0.2 mg/mL (Fig. 5C), and up to 17 when the concentration increased to 2.5 mg/mL (Fig. 5G). Based on 400 measurements from the cryo-TEM images of the onion-like dendrimersomes derived from (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a**, the average

spacing between the bilayers is 8.6 ± 0.6 nm. As shown by Fig. 4, the number of bilayers of the onion-like vesicles is proportional to their radius, which can be calculated by the equation $n = R/\sigma$, where N and R refer, respectively, to the number of bilayers and the radius of an individual onion-like vesicle, whereas σ is the average spacing between vesicle bilayers. This equation predicts the number of bilayers of the onion-like vesicles. Fig. 6A shows that the observed number of bilayers calculated according to this equation matches those of 21 onion-like vesicles determined visually from their cryo-TEM images (*SI Appendix, Fig. S3*). The diameter (D, Fig. 6B, *Inset*), the number of bilayers, and the PDI increase with the concentration of the Janus compound (Fig. 6B). The curves in Fig. 6B can be used as a calibration to predict the average number of bilayers of an onion-like dendrimersome at a specific concentration.

Single-single amphiphilic Janus dendrimers **9a**, **10a**, **11a**, **12a**, and **13a** (Fig. 2) also self-assemble into onion-like dendrimersomes in phosphate-buffered saline (PBS) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, as demonstrated by the assemblies generated from (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a** (*SI Appendix, Fig. S6*). At 0.5 mg/mL the onion-like dendrimersomes exhibited narrow PDI with the size of about 200 nm in PBS and Hepes buffers. These are suitable dimensions for cell uptake and drug delivery (26, 34, 48). The preparation of onion-like dendrimersomes by direct injection

Fig. 4. Relationship between diameter (nm) of individual onion-like dendrimersomes and their corresponding number of layers determined by cryo-TEM. Onion-like vesicles were self-assembled by (3,5)12G1-CH₂-NH-(3,4,5)-3EO-G1-(OCH₃)₃, **9a** (red $\blacktriangle$), (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a** (blue $\bullet$), and (3,5)12G1-CH₂-L-Ile-(3,4,5)-3EO-G1-(OCH₃)₃, **12a** (green $\blacksquare$).

Fig. 5. Cryo-TEM images of onion-like dendrimersomes self-assembled from (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a** in water at concentrations of (A) 0.025 mg/mL, (B) 0.1 mg/mL, (C) 0.2 mg/mL, (D) 0.5 mg/mL, (E) 1 mg/mL, (F) 2 mg/mL and (G) 2.5 mg/mL. The 3D surface plots generated by ImageJ at the bottom of each panel were calculated by transforming the grayscale intensity, or luminance of each pixel in the image into an effective height, with lower (darker) grayscale values being interpreted as greater heights. The detailed processing procedure is described in *Methods*.

of the solution of (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a** in various organic solvents into water was also investigated. Concentric onion-like vesicles were formed in nonalcohol solvent systems such as acetone, methyl ethyl ketone (MEK), 1,4-dioxane, and acetonitrile (*SI Appendix*, Fig. S7). Regardless of the nature of the solvent, it was determined by cryo-TEM that the thickness of the bilayer of the onion-like dendrimersomes was about 5.55 nm, which is almost equal to that of the bilayer determined by X-ray diffraction in bulk (*SI Appendix*, Table S3 and Fig. S11) (28). Less-regular shapes with nonuniform spacing between bilayers

were obtained from isopropanol and ethanol. With all solvents investigated except MEK, **10a** self-assembled into uniform onion-like dendrimersomes with the size ranging from 100 to 180 nm and PDI between 0.11 and 0.23 (*SI Appendix*, Table S3). Regular concentric onion-like dendrimersomes were also formed by injection of MEK solution into water. However, the resulting structures exhibited bimodal size distribution. It is therefore concluded that THF, acetone, acetonitrile, and 1,4-dioxane are the most suitable solvents for the direct injection method. This indicates the versatility of

Fig. 6. (A) Relationship between calculated and experimentally determined number of layers of onion-like dendrimersomes; (B) Concentration dependence of number of bilayers, diameter *D* (inset) and PDI of the onion-like dendrimersomes formed by (3,5)12G1-CH₂-L-Ala-(3,4,5)-3EO-G1-(OCH₃)₃, **10a**. PDI (yellow ▲), diameter (in nm, blue ■), and the number of bilayers (blue ●).

this method for the preparation of uniform onion-like dendrimersomes with submicrometer size.

Onion-like dendrimersomes were also prepared by injecting 1 mL of water or buffer into 50 μ L of THF solution (49). This method is referred as reverse injection. The resulting onion-like vesicles also exhibited narrow size distribution in water, PBS, and Hepes (*SI Appendix, Fig. S8*). At 0.5 mg/mL larger vesicles were obtained in water (185 nm) and Hepes (292 nm) by reverse injection, whereas onion-like vesicles with similar size (about 200 nm) were obtained in PBS by both reverse and direct injections. Both direct and reverse injection in the presence or absence of vortexing generated uniform onion-like vesicles (*SI Appendix, Fig. S10 and Table S4*). In both cases, a very small difference of PDI and vesicle size was observed in the absence of vortexing (*SI Appendix, Table S4*).

Conclusions

By using a constitutional isomeric library approach elaborated previously (28, 35), six primary structures of single–single amphiphilic Janus minidendrimers that self-assemble into uniform onion-like dendrimersomes by simple injection in water and buffers have been discovered. These onion-like vesicles were assembled by two extremely easy-to-handle direct and reverse injection methods. It has been demonstrated that the size and the number of bilayers of the onion-like vesicles are predictable by the final concentration of the Janus dendrimer. At low-concentration, double-bilayer vesicles are formed, whereas at higher concentrations they form multibilayer onion-like vesicles. The onion-like dendrimersomes provide simple and easily accessible mimics of various biological membranes of different cells and organelles such as Gram-negative bacteria (2, 3), cell nuclei (4), and multilamellar bodies (11, 12). The direct and reverse injection method has been proven versatile and reliable with various solvents such as THF, acetone, acetonitrile, and 1,4-dioxane. In addition, the assembly of onion-like vesicles reported here could become a promising tool for the construction of time-dependent delivery devices containing multiple water-insoluble and water-soluble cargos incorporated in different bilayers and in between different bilayers of these architectures. Research on this concept, on the discovery of additional primary structures assembling in onion-like vesicles including dendrimersomes, and on the elucidation of the mechanism of self-assembly, is in progress.

Methods

Onion-like dendrimersomes were prepared by the following general method. A stock solution was prepared by dissolving the required amount of amide containing single–single amphiphilic Janus minidendrimer in freshly distilled THF (or other water-soluble solvent) at 23 °C. Onion-like dendrimersomes were generated by the injection of 100 μ L of stock solution into 2 mL of Millipore water followed by 5-s vortexing to give the final dendrimer concentration of 0.025–4 mg/mL in water or in PBS and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffers. Different final concentrations of onion-like dendrimersomes in water were obtained by varying the concentration of the stock solution followed by quick injection (0.3–0.5 s) of 100 μ L of the stock solution into 2.0 mL of Millipore water.

DLS was performed with a Malvern Instruments particle sizer (Zetasizer Nano S, Malvern Instruments) equipped with a He–Ne laser (4 mW) of 633 nm and avalanche photodiode positioned at 175° to the beam and temperature-controlled cuvette holder. Instrument parameters were determined automatically along with measurement times. All experiments were performed in triplicate.

Cryo-TEM was performed on a JEOL 2100 TEM operating at a voltage of 200 kV. This instrument is equipped with a Gatan Peltier cooled CCD imaging system. For sample preparation, a copper TEM grid (300-mesh precoated with lacy carbon film, Ted Pella) was treated with oxygen and hydrogen plasma (Gatan 950 Solarus plasma cleaner) to create a hydrophilic surface, and then a droplet of 2 μ L solution was pipetted onto the grid loaded into a Gatan CryoPlunge 3 (Cp3) apparatus by nonmagnetic tweezers. Each grid was blotted for 1.5 s to obtain a thin solution layer with thickness ranging from ~100 to ~350 nm (see also the discussion of Fig. 5 and *SI Appendix, Fig. S9*). The sample was allowed to relax for ~10 s to remove any residual stress imparted by blotting before quickly plunging into liquefied ethane (~–180 °C) cooled by a reservoir of liquid nitrogen to ensure the vitrification of water. The vitrified samples were transferred to a Gatan CT3500TR Single Tilt Cryo Transfer Holder in a cryo-transfer stage immersed in liquid nitrogen. During the imaging, the cryo-holder was kept below –170 °C to prevent sublimation of vitreous solvent. A Gatan low-dose CCD camera recorded the digital images. The 3D surface plots of the onion-like dendrimersomes were made from their cryo-TEM images by using ImageJ (v1.46r) (50). In ImageJ, the original cryo-TEM images were first converted from red, green, blue format into 8-bit grayscale format. Subsequently, the grayscale intensity was adjusted using the “Threshold” function, and the contrast was enhanced and normalized, so as to clearly visualize the bilayer boundaries. The 3D surface plots were generated using the “interactive 3D surface plot” plugin preloaded in ImageJ. This plugin converts the pixel values (luminance) into height information, with darker areas (lower grayscale values) corresponding to greater heights. Finally, a smoothing function was applied to reduce noise.

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