

Self-Assembly of Glycerol-Amphiphilic Janus Dendrimers Amplifies and Indicates Principles for the Selection of Stereochemistry by Biological Membranes

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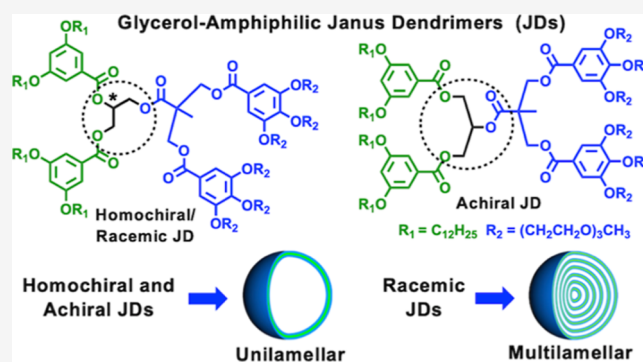
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ABSTRACT: The principles for the selection of the stereochemistry of phospholipids of biological membranes remain unclear and continue to be debated. Therefore, any new experiments on this topic may help progress in this field. To address this question, three libraries of constitutional isomeric glycerol-amphiphilic Janus dendrimers (JDs) with nonsymmetric homochiral, racemic, and symmetric achiral branching points were synthesized by an orthogonal–modular–convergent methodology. These JDs amplify self-assembly, and therefore, monodisperse vesicles known as dendrimersomes (DSs) with predictable dimensions programmed by JD concentration were assembled by rapid injection of their ethanol solution into water. DSs of homochiral JD enantiomers, racemic, including mixtures of different enantiomers, and achiral exhibited similar DS size-concentration dependence. However, the number of bilayers of DSs assembled from homochiral, achiral, and racemic JDs determined by cryo-TEM were different. Statistical analysis of the number of bilayers and coarse-grained molecular dynamics simulations demonstrated that homochiral JDs formed predominantly unilamellar DSs. Symmetric achiral JDs assembled only unilamellar DSs while racemic JDs favored multilamellar DSs. Since cell membranes are unilamellar, these results indicate a new rationale for nonsymmetric homochiral *vs* racemic selection. Simultaneously, these experiments imply that the symmetric achiral lipids forming more stable membrane, probably had been the preferable assemblies of prebiotic cell membranes.



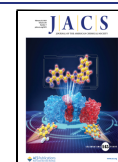
INTRODUCTION

Homochirality is an essential signature of living matter. During the evolution of life, nature selected homochirality for most biological molecules and macromolecules.¹ L-Amino acids and D-sugars have been selected as the main components of biological systems.² However, phospholipids, the backbone of biological membranes, display different chirality in different living systems.³ Natural phospholipids are almost ubiquitously homochiral. Nevertheless, a case of dual homochirality is also known for phospholipids. In detail, membranes of archaea are based on *sn*-glycerol-1-phosphate (G1P) enantiomers with *S* configurational stereochemistry, while membranes of bacteria and eukaryotes employ exclusively *sn*-glycerol-3-phosphate (G3P) enantiomers with *R* configurational stereochemistry.^{3a,c,4} G1P and G3P exhibit opposite handedness, but both of them provide efficient membranes. The phenomenon that different phospholipid homochiralities exist in nature is known as the lipid divide. It is believed that archaea and bacteria which are the two basal domains of life as well as eukarya have a common ancestor, referred to as the last universal common ancestor (LUCA).^{3,4}

However, the chirality of the LUCA's membrane (homochiral or heterochiral) and how G1P and G3P evolve from the LUCA's membrane remain unknown, although many theories and hypotheses toward these issues have been advanced.^{4,5} One theory pointed out that the LUCA's membrane was heterochiral or racemic, and the instability between the two phospholipid enantiomers eventually resulted in the lipid divide.^{5b} Nevertheless, a recent study found that the robustness of cells with a hybrid heterochiral lipid membrane is higher than that with a homochiral lipid membrane.^{5d} Another theory stated that the LUCA's membrane could be homochiral as it was enzymatically synthesized. However, the controversial question is what homochirality was LUCA's membrane? G1P

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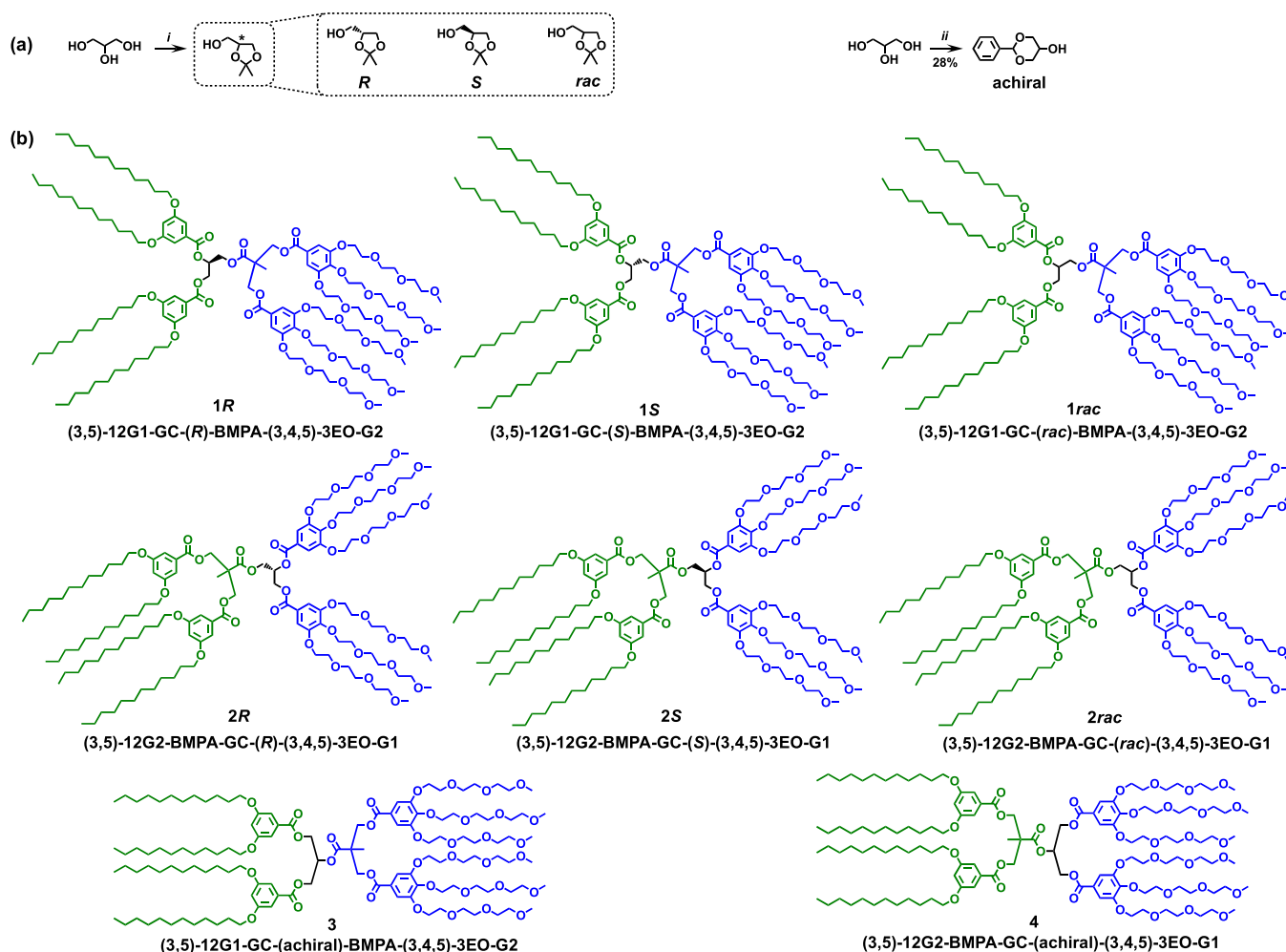


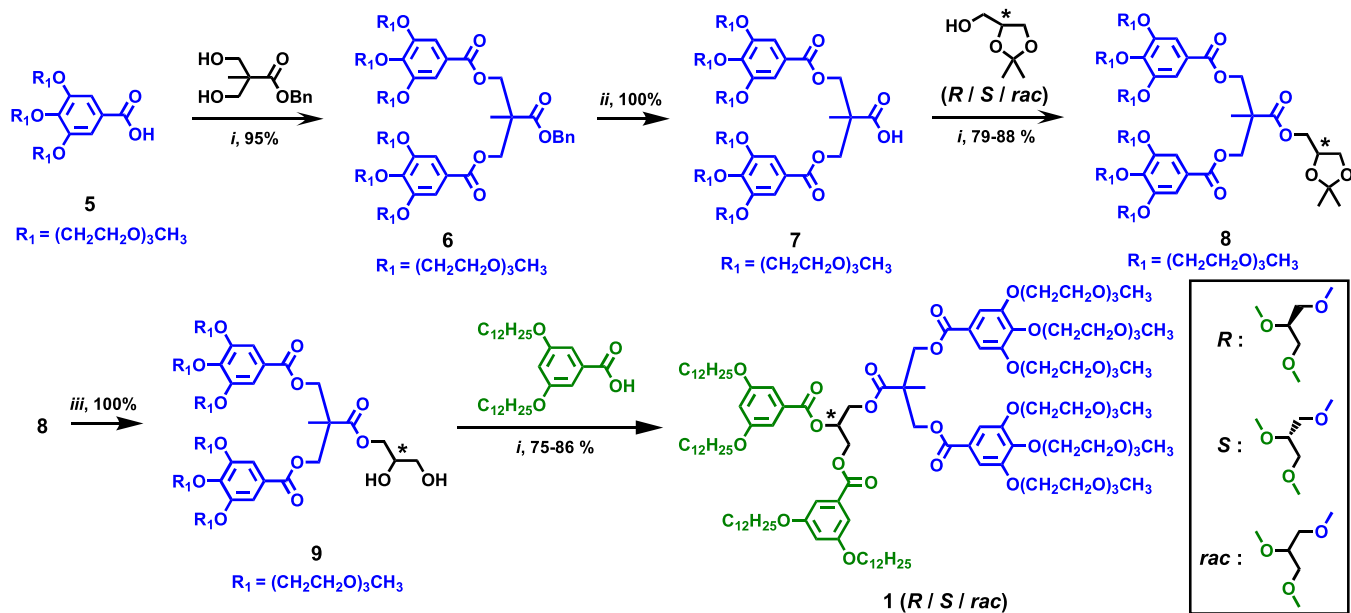
Figure 1. (a) Synthesis and nonsymmetric structures of *R*-, *S*-, and *rac*- and symmetric achiral glycerols with two protected OH. Reagents and conditions: (i) PTSA-H₂O, 4 Å MS, hexane/acetone, Dean-Stark, reflux, and 12 h and (ii) benzaldehyde, H₂SO₄, toluene, Dean-Stark, reflux, and 6 h. (b) Structures of nonsymmetric homochiral (*1R*, *1S*, *2R*, and *2S*) and racemic (*1rac* and *2rac*) and symmetric achiral (*3* and *4*) glycerol JDs.

or G3P? According to the literature results, both of them have been proposed and supported by different research groups.^{4,5c} In addition to homochiral or heterochiral possibilities, the LUCA's membrane could also be achiral. We hypothesize that the LUCA's membrane may be composed of achiral lipids, but they disappeared during the evolution of cellular life. This is probably due to the appearance of homochiral proteins including enzymes for the synthesis of membrane lipids and membrane proteins, resulting in the selectivity of homochiral lipids in biological membranes and only one chirality of lipids existing in one type of membranes.

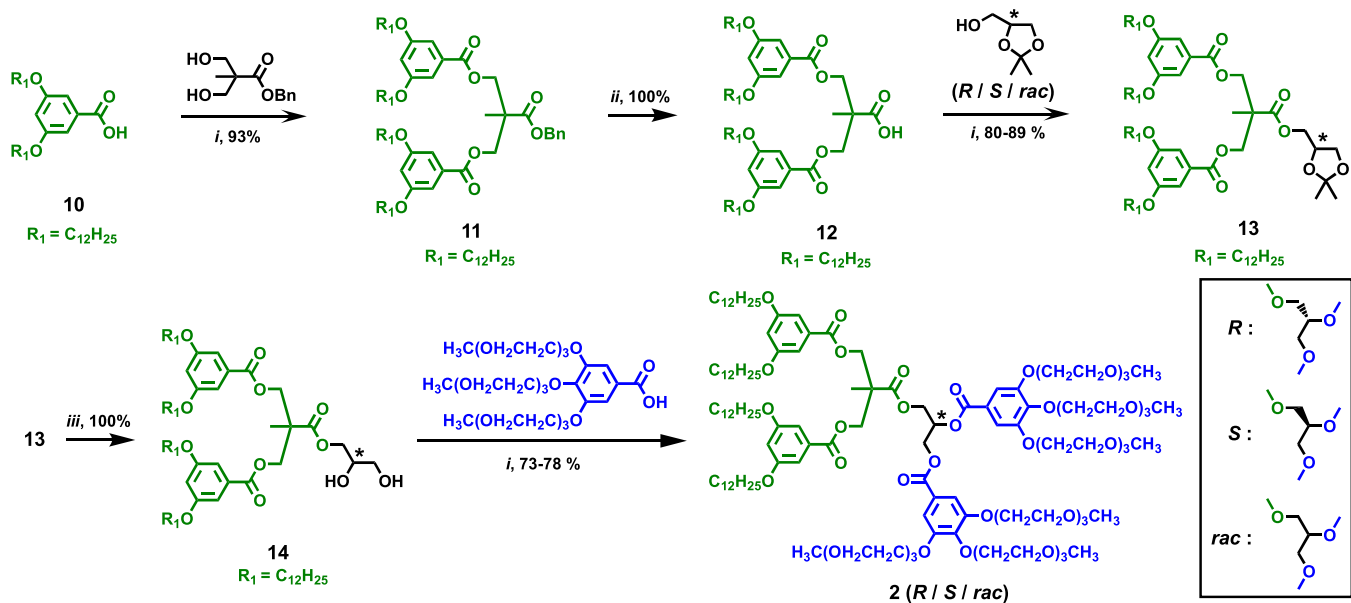
Liposomes, an important class of biological assemblies, represent the basis of cell membranes. Synthetic variants, including synthetic liposomes,⁶ stealth liposomes,⁷ and polymersomes,⁸ have been elaborated to mimic natural cells. In addition to being models for biological membranes, synthetic liposomes and vesicles have been used to deliver active agents such as drugs and genes *in vitro* and *in vivo*.⁹ Liposomes assembled from phospholipids are unstable because the alkene units in the hydrophobic tails are readily oxidized and exhibit poor mechanical properties. Stealth liposomes co-assembled from phospholipids, phospholipids conjugated with poly(ethylene glycol) (PEG) and cholesterol, are more stable but are polydisperse requiring time-consuming fractionation to desirable dimensions and narrow polydispersity. Polymersomes

assembled from amphiphilic block copolymers exhibit excellent stability but are not always biocompatible, and their membrane thickness is wider than that of cell membranes.⁸

Our laboratory developed a class of vesicles named dendrimersomes (DSs), which are assembled from amphiphilic Janus dendrimers (JDs).¹⁰ DSs exhibit better stability in time than liposomes, can be functionalized on their surface through their multivalency both in the hydrophilic and hydrophobic parts of JDs, and display similar membrane thickness.¹¹ Amphiphilic JDs with sugars conjugated on their hydrophilic part denoted Janus glycodendrimers (JGDs) self-assemble into vesicles known as glycodendrimersomes (GDSs), which mimic the glycan of biological membranes and bind sugar-binding proteins.¹² Recently, our group developed a new class of JDs, ionizable amphiphilic Janus dendrimers (IAJDs), which showed great potential in targeted delivery of messenger RNA (mRNA) *in vitro* and *in vivo*.¹³ As a new synthetic delivery vector with high transfection efficiency of mRNA and high stability, IAJD facilitates the development of new mRNA vaccines and drugs. DSs are generally assembled by injection of their JD prepared in a water-miscible solvent solution such as ethanol or THF into water or buffer. This simple self-assembly procedure provides monodisperse, impermeable, and stable vesicles with excellent mechanical properties.

Scheme 1. Synthesis of Nonsymmetric Glycerol JDs of Library 1^a

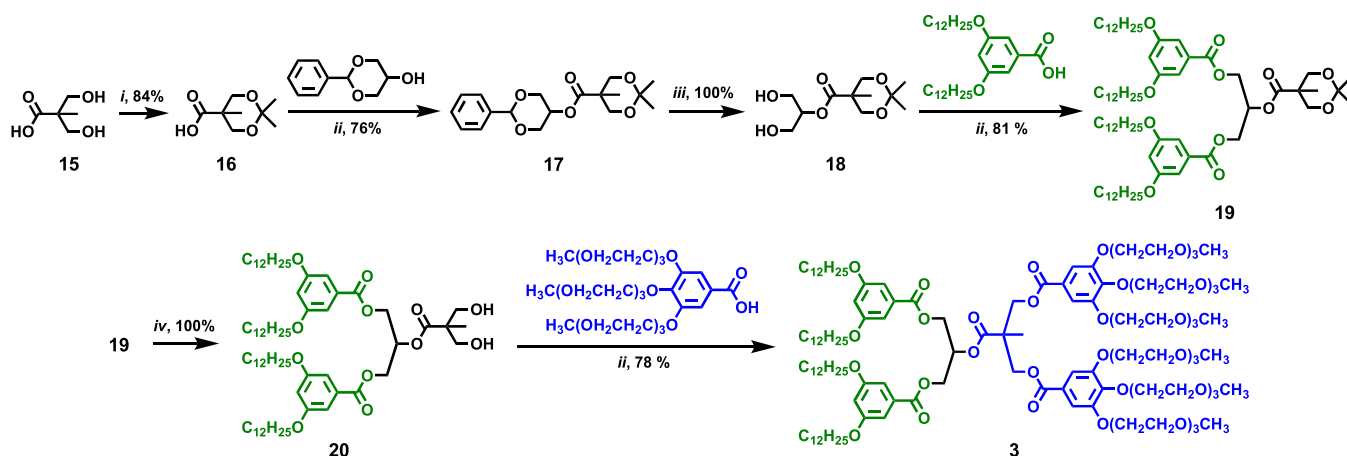
^aReagents and conditions: (i) DCC, DPTS, DCM, 23 °C, and 12 h; (ii) H₂, Pd/C, EtOAc, 23 °C, and 8 h; and (iii) 1 M HCl, MeOH, 23 °C, and 1 h.

Scheme 2. Synthesis of Nonsymmetric Glycerol JDs of Library 2^a

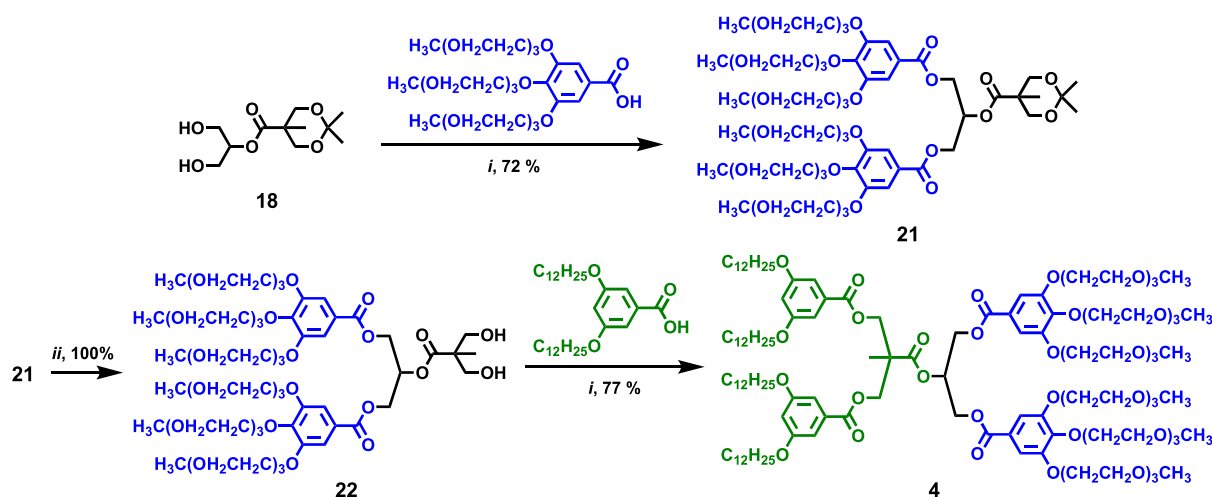
^aReagents and conditions: (i) DCC, DPTS, DCM, 23 °C, and 12 h; (ii) H₂, Pd/C, EtOAc, 23 °C, and 8 h; and (iii) 1 M HCl, 1,4-dioxane, 60 °C, and 8 h.

The properties of DSs rely on the architecture of the assembling amphiphilic JDs. JDs assembling DSs were classified into “Twin–Twin” and “Single–Single.” “Twin–Twin” JDs are constructed by combinations of twin-hydrophobic and twin-hydrophilic dendrons with an achiral pentaerythritol (PE) or tris(hydroxymethyl)aminomethane (Tris) core, while “Single–Single” JDs are made from single-hydrophobic and single-hydrophilic dendrons connected by an ester or an amide linker. Unilamellar and onion-like DSs have been discovered and designed, and their dimensions are predicted^{10f} by self-assembly of “Twin–Twin” and “Single–Single” JDs.¹⁴ However, there is no report on amphiphilic JDs

with a chiral, racemic, or achiral glycerol core connecting hydrophilic and hydrophobic parts. Nature selects homochiral rather than racemic but not achiral glycerol phospholipids to construct biological membranes. In this publication, we address the very simple question: Why homochiral and not racemic or achiral lipids are used by contemporary cell membranes in biology? Only because they are enzymatically produced? What is the difference between the prebiotic primitive achiral lipids forming biological membranes and their contemporary chiral lipids?

Scheme 3. Synthesis of Symmetric Achiral Glycerol JD 3^a

^aReagents and conditions: (i) PTSA·H₂O, 2,2-dimethoxypropane, acetone, 23 °C, and 3 h; (ii) DCC, DPTS, DCM, 23 °C, and 12 h; (iii) H₂, Pd/C, EtOAc, 23 °C, and 8 h; and (iv) 1 M HCl, 1,4-dioxane, 60 °C, and 8 h.

Scheme 4. Synthesis of Symmetric Achiral Glycerol JD 4^a

^aReagents and conditions: (i) DCC, DPTS, DCM, 23 °C, and 12 h and (ii) 1 M HCl, MeOH, 23 °C, and 1 h.

RESULTS AND DISCUSSION

Orthogonal Modular Convergent Synthesis of Three Libraries of Homochiral, Racemic, and Achiral Glycerol-Based JDs. To address the question of biological homochirality of cell membranes, we investigated the self-assembly of all constitutional isomeric glycerol JDs that facilitate the construction of homochiral, racemic, both nonsymmetric, and achiral, symmetric, structures via the stereochemistry of their branching point (Figure 1a). These JDs were expected to provide an amplification of the principles employed by phospholipids during their self-assembly and during the selection of their homochirality. Eight JDs including four homochiral, two racemic, and two achiral were synthesized (Figure 1b). Injection of their ethanol solution into water produced DSs with different dimensions and morphologies. They were characterized by dynamics light scattering (DLS) and cryogenic transmission electron microscopy (cryo-TEM).

The three libraries of glycerol-based JDs synthesized by an orthogonal–modular–convergent methodology are shown in Figure 1a,b. Both libraries 1 (top of Figure 1b) and 2 (middle of Figure 1b) contain two JDs with a nonsymmetric homochiral glycerol branching point 1R, 1S in library 1 and

2R, 2S in library 2, and one JD with a racemic branching point per library 1rac, 2rac (Figure 1b). Library 3 contains two constitutional isomeric symmetric achiral glycerol JDs (bottom of Figure 1b). The main building blocks employed in the orthogonal–modular–convergent synthesis include the hydrophilic acid 5 (Scheme 1), the hydrophilic second-generation acid 7 (Scheme 1), the hydrophobic acid 10 (Scheme 2), the hydrophobic second-generation acid 12 (Scheme 2), the *R/S*-*rac*-2,2-dimethyl-1,3-dioxolane-4-methanol (isopropylidene glycerol) containing the stereocenters, and the achiral 5-hydroxy-2-phenyl-1,3-dioxane (Figure 1a and Scheme 3).

The hydrophilic acid 5 was synthesized by introducing triethylene glycol monomethyl ether in the 3,4,5-positions of the methyl ester of gallic acid, followed by hydrolysis of the ester group (Scheme S1). Two hydrophilic acids 5 were esterified by a 2,2-bis(hydroxymethyl)propionate linker to provide the hydrophilic second-generation acid 7 (Scheme 1). Similarly, the hydrophobic acid 10 was synthesized by attaching dodecyl groups on the 3,5-positions of 3,5-dihydroxybenzoic acid.^{10g} The hydrophobic second-generation acid 12 was prepared by connecting two hydrophobic acids 10 with a 2,2-bis(hydroxymethyl)propionate linker (Scheme 2).

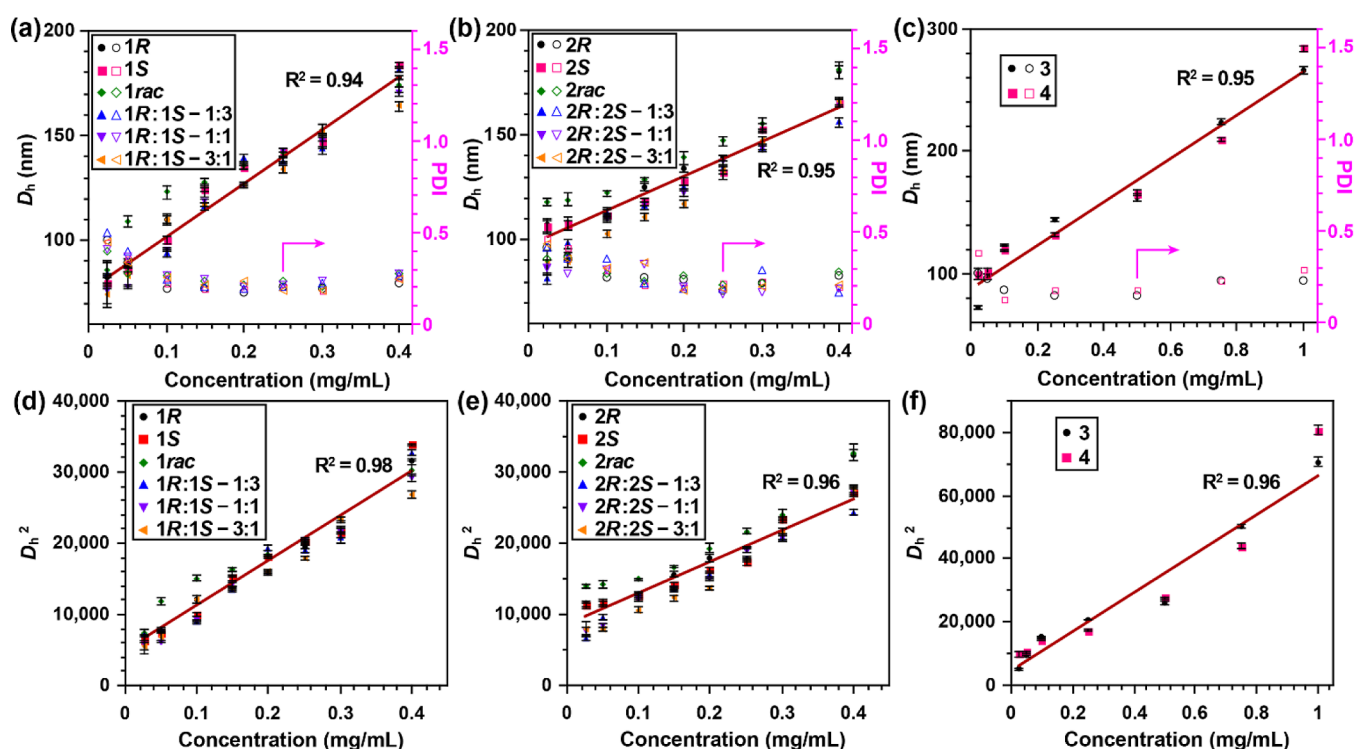


Figure 2. Concentration dependence of diameter (D_h , in nm) and square diameter (D_h^2) of DSs assembled by nonsymmetric and symmetric glycerol JDs in water. (a,d) Correspond to glycerol JDs of library 1. (b,e) Correspond to glycerol JDs of library 2. (c,f) Correspond to achiral glycerol JDs 3 and 4.

Table 1. Dimensions of Assemblies from Nonsymmetric Glycerol JDs in Library 1 at Different Concentrations of JDs in Water (Size Distribution Is Recorded with Intensity; Sample Number $N = 3$ for Each Group)

concentration of JD (mg·mL ⁻¹)	1R		1S		1rac		1R:1S—1:3		1R:1S—1:1		1R:1S—3:1	
	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI
0.5	233 ± 4	0.326	229 ± 5	0.438	281 ± 3	0.240	179 ± 2	0.421	184 ± 1	0.271	266 ± 3	0.376
0.4	178 ± 1	0.228	184 ± 1	0.258	174 ± 3	0.275	181 ± 1	0.268	171 ± 2	0.274	164 ± 2	0.246
0.3	148 ± 1	0.190	147 ± 3	0.186	153 ± 2	0.203	144 ± 3	0.226	147 ± 2	0.236	153 ± 2	0.180
0.25	140 ± 2	0.202	142 ± 2	0.203	142 ± 2	0.238	138 ± 1	0.207	143 ± 1	0.214	134 ± 2	0.186
0.2	135 ± 1	0.175	135 ± 1	0.195	136 ± 2	0.216	139 ± 2	0.197	126 ± 1	0.201	127 ± 1	0.234
0.15	124 ± 1	0.195	124 ± 3	0.190	128 ± 2	0.232	116 ± 2	0.206	119 ± 1	0.247	117 ± 1	0.210
0.1	110 ± 3	0.197	100 ± 2	0.223	123 ± 3	0.268	94 ± 2	0.243	96 ± 1	0.269	110 ± 2	0.257
0.05	87 ± 2	0.336	86 ± 1	0.359	109 ± 3	0.277	89 ± 2	0.401	78 ± 1	0.368	83 ± 5	0.334
0.025	82 ± 2	0.455	80 ± 3	0.446	86 ± 4	0.398	84 ± 5	0.502	76 ± 8	0.410	74 ± 4	0.451

Table 2. Dimensions of Assemblies from Nonsymmetric Glycerol JDs in Library 2 at Different Concentrations of JDs in Water (Size Distribution Is Recorded with Intensity; Sample Number $N = 3$ for Each Group)

concentration of JD (mg·mL ⁻¹)	2R		2S		2rac		2R:2S—1:3		2R:2S—1:1		2R:2S—3:1	
	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI	D_h (nm)	PDI
0.5	319 ± 4	0.497	288 ± 5	0.295	412 ± 13	0.449	304 ± 3	0.511	324 ± 6	0.452	390 ± 10	0.371
0.4	180 ± 3	0.263	165 ± 2	0.205	181 ± 4	0.283	156 ± 2	0.170	165 ± 2	0.195	166 ± 2	0.218
0.3	152 ± 1	0.227	153 ± 2	0.235	155 ± 3	0.225	144 ± 2	0.295	144 ± 1	0.175	147 ± 2	0.208
0.25	139 ± 1	0.186	132 ± 3	0.223	147 ± 2	0.211	134 ± 1	0.192	138 ± 2	0.158	133 ± 2	0.193
0.2	134 ± 2	0.242	128 ± 2	0.203	139 ± 3	0.260	125 ± 1	0.189	122 ± 1	0.205	117 ± 2	0.188
0.15	125 ± 2	0.257	119 ± 1	0.212	129 ± 1	0.236	116 ± 2	0.223	117 ± 1	0.324	111 ± 2	0.335
0.1	111 ± 2	0.258	112 ± 2	0.291	122 ± 1	0.277	113 ± 2	0.359	110 ± 2	0.293	103 ± 2	0.305
0.05	108 ± 3	0.368	107 ± 2	0.422	119 ± 3	0.365	98 ± 2	0.359	92 ± 2	0.280	89 ± 2	0.361
0.025	107 ± 3	0.416	106 ± 3	0.465	118 ± 2	0.361	81 ± 2	0.419	87 ± 3	0.309	89 ± 6	0.439

For library 1 (Scheme 1), the hydrophilic second-generation methanol, followed by deprotection to generate the corresponding diol 9. The esterification of this hydrophilic diol and

methanol, followed by deprotection to generate the corresponding diol 9. The esterification of this hydrophilic diol and

two hydrophobic first-generation acids produced **1R**, **1S**, and **1rac** in 75–86% isolated yield.

The synthesis of **2R**, **2S**, and **2rac** of library 2 (Scheme 2) was accomplished by the esterification performed with the hydrophobic second-generation acid and the second esterification with the hydrophobic diol **14** and two hydrophilic acids **5** in 73–78% isolated yield. Schemes 3 and 4 outline the synthesis of the achiral JDs **3** and **4**. The achiral branching core **17** was synthesized by the esterification of **16** with 5-hydroxy-2-phenyl-1,3-dioxane to produce diol **18** after deprotection of the benzylidene acetal group. The achiral JDs **3** and **4** were synthesized by attaching two hydrophilic acids **5** and two hydrophobic acids **10** to diol **18** (Schemes 3 and 4). The detailed synthesis and characterization are available in the Supporting Information.

Self-Assembly of Glycerol JDs into DSs. The self-assembly of all eight glycerol JDs was performed by injection of their ethanol solution (50 μ L) into 1 mL of Milli-Q water. DSs are kinetically controlled assemblies. The size distribution and structure of the resulting DSs were analyzed by DLS (Figure 2) and cryo-TEM. Previously, we found that unilamellar and onion-like DSs assembled from “Twin–Twin” and “Single–Single” JDs displayed a size-concentration dependence.^{10f,14b} In a certain concentration range, the size of DSs increased with the concentration of JDs injected in water or buffer. A similar size-concentration dependence was observed for glycerol JDs (Figure 2). The detailed experimental results are summarized in Tables 1–3, and representative DLS curves are shown in

the size of the resulting DSs increased with the concentration of JDs in water for **1R**, **1S**, and **1rac** for concentration range from 0.025 to 0.5 $\text{mg}\cdot\text{mL}^{-1}$. The dimensions of the assemblies were identical when the concentrations of the JDs in water were identical, and the linear fitting line of the size-concentration dependence represented this identical trend. Sizes of assemblies from mixtures of **1R** and **1S** in different ratios (1:3, 1:1, and 3:1) were also identical (Figure 2a). This indicated that sizes of assemblies from JD homochiral enantiomers (**1R** and **1S**) and racemic mixtures with different ratios of enantiomers were identical at the same concentrations of JDs in water. This is expected since, with the exception of the rotation of polarized light, the physical properties of enantiomers and of their racemic mixtures must be identical. In addition, the square of the diameter of DSs displayed a linear dependence on concentration in the range from 0.025 to 0.4 for homochiral and racemic and to 1.0 $\text{mg}\cdot\text{mL}^{-1}$ for achiral JDs (Figure 2d,f). This result is consistent with the size-concentration relationship of JDs already reported^{10f,14b} and helps predict the dimensions of DSs with the aid of JD concentrations.

2R, **2S**, and **2rac** as well as mixtures of **2R** and **2S** with different molar ratios (1:3, 1:1, and 3:1) showed a similar size-concentration dependence (Figure 2b). The diameter rather than the square of diameter of DSs displayed a linear relationship vs concentration of glycerol JD in the concentration range from 0.025 to 0.4 for homochiral and racemic and to 1.0 $\text{mg}\cdot\text{mL}^{-1}$ for achiral JDs (Figure 2b,c,e). In addition, the representative dependence of polydispersity index (PDI) on diameter (D_h , in nm) for DSs assembled by glycerol JDs **1R**, **2R**, and **3** is shown in Figure 4, in which we can see when the sizes of DSs are larger than 120 nm, the PDI is smaller than 0.3, while the PDI increases to around 0.4 when the sizes are smaller than 120 nm, which may result in the deviation of linear size-concentration dependence when the concentration of JDs is lower than 0.1 $\text{mg}\cdot\text{mL}^{-1}$.

Determination of the Structure of Homochiral, Racemic, and Achiral DSs by Cryo-TEM. Although dimensions do not show differences when comparing homochiral, achiral, and racemic JDs, the structure of the resulting DSs was investigated by cryo-TEM. Substantial differences were observed during cryo-TEM investigations. cryo-TEM of enantiomers **2R** and **2S** (Figure 5a,b) showed unilamellar DSs predominantly, being accompanied by a small amount of multilamellar DSs. The cryo-TEM images of glycerol JD **2rac** (Figure 5c) showed the opposite result, in

Table 3. Dimensions of Assemblies from Symmetric Achiral Glycerol JDs **3 and **4** at Different Concentrations of JDs in Water (Size Distribution Is Recorded with Intensity; Sample Number $N = 3$ for Each JD)**

concentration of JD ($\text{mg}\cdot\text{mL}^{-1}$)	3		4	
	D_h (nm)	PDI	D_h (nm)	PDI
1.0	266 ± 3	0.233	284 ± 3	0.291
0.75	224 ± 2	0.236	210 ± 2	0.233
0.5	162 ± 3	0.152	166 ± 2	0.183
0.25	144 ± 1	0.153	132 ± 2	0.180
0.1	123 ± 1	0.183	119 ± 1	0.136
0.05	97 ± 2	0.241	102 ± 2	0.266
0.025	72 ± 2	0.272	100 ± 5	0.391

Figure 3. The derived count rates of all DSs are between 150 and 500 kilo counts per second (kcps). Figure 2a shows how

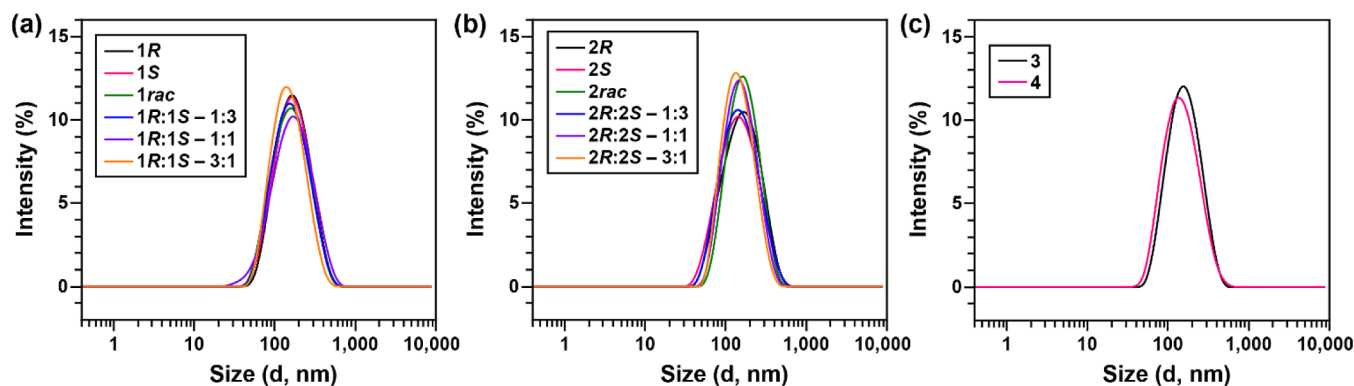


Figure 3. Representative DLS curves of assemblies from glycerol JDs in library 1 (a), library 2 (b), and achiral JDs **3** and **4** (c) with the same concentration of JD (0.25 $\text{mg}\cdot\text{mL}^{-1}$) in water (size distribution is recorded with intensity).

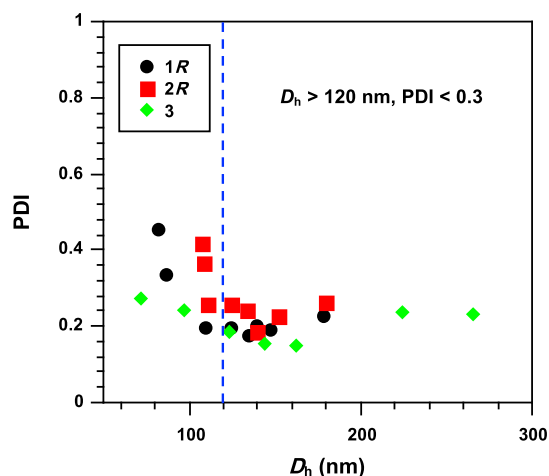


Figure 4. Dependence of PDI on diameter (D_h , in nm) for DSs assembled by glycerol JDs **1R**, **2R**, and **3**. All DSs show a PDI lower than 0.3 when the corresponding D_h is larger than 120 nm.

which multilamellar DSs were predominant while unilamellar DSs were observed in a small amount. The red numbers in Figure 5 stand for the unilamellar DSs, while the yellow numbers for multilamellar DSs. The calculated percentages of unilamellar DSs in the selected images for **2R**, **2S**, and **2rac** are 91% (11 DSs counted), 70% (40 DSs counted), and 13% (48 DSs counted), respectively. The same trend was found for cryo-TEM of **1R**, **1S**, and **1rac** (Figures S23–S25). This indicates that homochiral JDs tend to self-assemble predominantly into unilamellar DSs, while racemic favor the formation of multilamellar DSs. Achiral DSs from glycerol JDs **3** and **4** are all unilamellar (Figures Sd–f and S29 and S30). In

addition, the 3D surface plots of vesicles in the corresponding cryo-TEM images further prove this phenomenon. As shown in Figure 6a–d, homochiral JDs **1R**, **1S**, **2R**, and **2S** self-assemble into unilamellar DSs, while racemic JDs **1rac** and **2rac** self-assemble into onion-like multilamellar DSs (Figure 6e–g). Achiral JDs **3** and **4** self-assemble into unilamellar DSs (Figure 6h,i), which is similar to homochiral JDs, but they exhibit more solid membranes compared to those assembled by **1R**, **1S**, **2R**, and **2S** JDs.

Statistical Analysis of the Structure of Homochiral, Racemic, and Achiral DSs. Statistical analysis was used to organize the number of bilayers from cryo-TEM. The distribution of the number of lamellae in DSs was analyzed for each sample to construct the respective histograms (Figure 7). The histograms represent the distribution function of probability. The number of bilayers of racemic JDs (**1rac** and **2rac**) exhibit a near-normal distribution (Gaussian) as demonstrated by various statistic tests. However, the enantiomerically pure JDs (**1R**, **1S**, **2R**, and **2S**) exhibit Chi-squared distribution (χ^2 distribution) with a shape parameter of 1 or 2. The difference in the distributions of number of bilayers for homochiral *vs* racemic glycerol-based JDs emphasizes the difference between the self-assembly structures, unilamellar *vs* multilamellar.

To investigate the normality of the distribution of racemic JDs, we used SPSS statistics software, which utilized normality tests of Kolmogorov–Smirnov and Shapiro–Wilk. Both methods demonstrated that the distribution of racemic JDs (**1rac** and **2rac**) were normal-like. For a more detailed study of distribution functions, we calculated the skewness (third standardized moment and a measure of the asymmetry) and kurtosis (fourth standardized moment and a measure of the extreme values in either tail) of the data (number of DS layers,

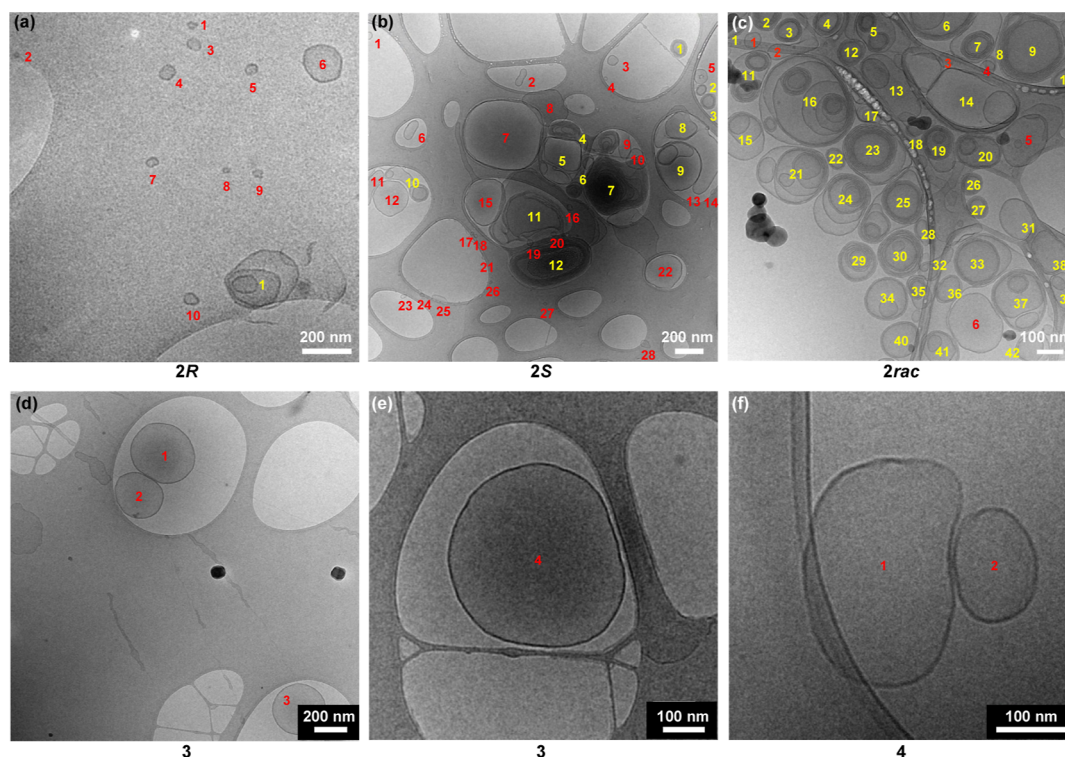


Figure 5. Selected cryo-TEM images of DSs assembled by glycerol JDs **2R** (a), **2S** (b), **2rac** (c), achiral **3** (d,e), and achiral **4** (f). The concentration of glycerol JDs was 1 mg·mL^{−1}.

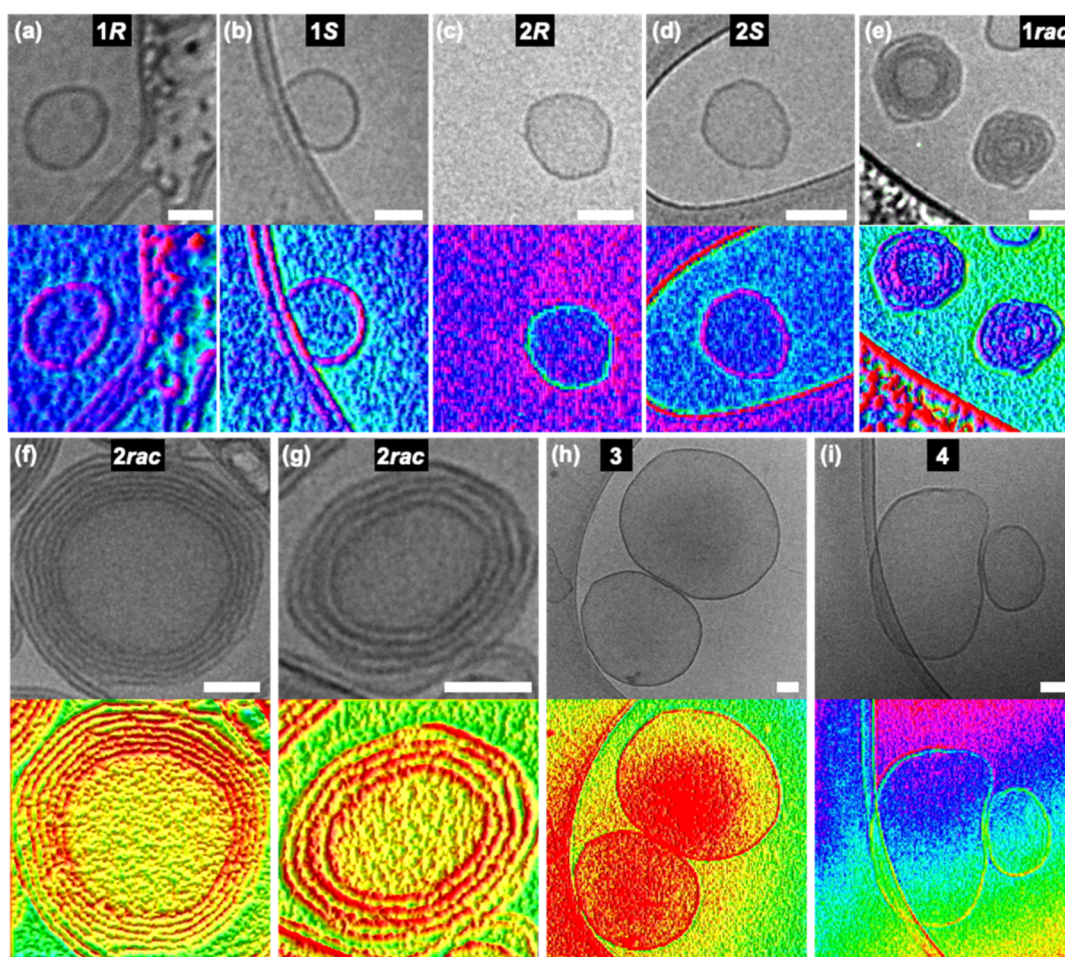


Figure 6. Representative cryo-TEM images (the upper half part in the panel) and 3D surface plots (the lower half part in the panel) of DSs assembled by glycerol JDs. (a) Corresponds to the DS assembled by **1R**. (b) Corresponds to the DS assembled by **1S**. (c) Corresponds to the DS assembled by **2R**. (d) Corresponds to the DS assembled by **2S**. (e) Corresponds to the DS assembled by **1rac**. (f,g) Correspond to the DSs assembled by **2rac**. (h) Corresponds to the DS assembled by **3**. (i) Corresponds to the DS assembled by **4**. The scale bar is 50 nm. The 3D surface plots were generated by ImageJ software through transforming the pixel values (luminance) into height information, with darker areas (lower grayscale values) corresponding to greater heights.

Tables S1 and S2). The skewness of the distributions of the number of bilayers for **1rac** and **2rac** is slightly positive (0.70 and 0.58, respectively). Positive skewness means the tails are extending to the right. These relatively low values are in line with the previous conclusion that their distribution is normal-like. Comparing the skewness of the distribution of racemic JDs with homochiral JDs shows that **1R**, **1S**, **2R**, and **2S** are very positively skewed (1.977, 1.687, 3.313, and 3.029, respectively) and nonsymmetric. This difference in distribution parameter (skewness) strongly supports the difference between homochiral (unilamellar) and racemic (multilamellar) glycerol-based JDs.

The second distribution parameter which we have investigated is the kurtosis and excess kurtosis (kurtosis -3). For normal distribution, the kurtosis is 3, and the excess of kurtosis is zero. The distributions of racemic JDs have negative kurtosis (-1.623 for **1rac** and -0.423 for **2rac**), indicating that the peak is lower than the peak of a normal distribution. While homochiral JDs show distributions with positive kurtosis (3.932 for **1R**, 2.797 for **1S**, 10.980 for **2R**, and 9.502 for **2S**), indicating that the corresponding peaks are higher than those of the normal distribution. This result highlights the difference

between the number of bilayers of DSs assembled from homochiral and racemic glycerol JDs.

Coarse-Grained Molecular Dynamics Simulations of Homochiral, Racemic, and Achiral DSs. To investigate the chirality effect on the morphological and topological changes in JD aggregates, we conducted coarse-grained (CG) molecular dynamics (MD) simulations for large aggregates composed of 20,000 JD molecules using the SPICA force field.¹⁵ The simulation protocol here is the same as that we used for vesicle formation simulation.¹⁵ First, JDs were randomly arranged in a cubic simulation box and hydrated with approximately 50 CG water particles per JD molecule (Figure 8a). Then, the JD aggregates obtained after energy minimization and 50 ns equilibration MDs were placed into a larger water box. At this stage, the CG water per JD molecule was increased to approximately 245 particles. Then, to monitor the development of morphology and topology of JD aggregates in dilute solutions, we performed 2 μ s MDs for glycerol JDs **2R** and **2rac** aggregate systems, while we ran 1.5 μ s MDs for achiral JD **3** and **4** aggregates. Figure 8b displays the final configurations obtained from the MDs. The maximum radii of glycerol JDs **2R**, **3**, and **4** aggregates were estimated to be approximately 40 nm, which is larger than that of the JD **2rac**

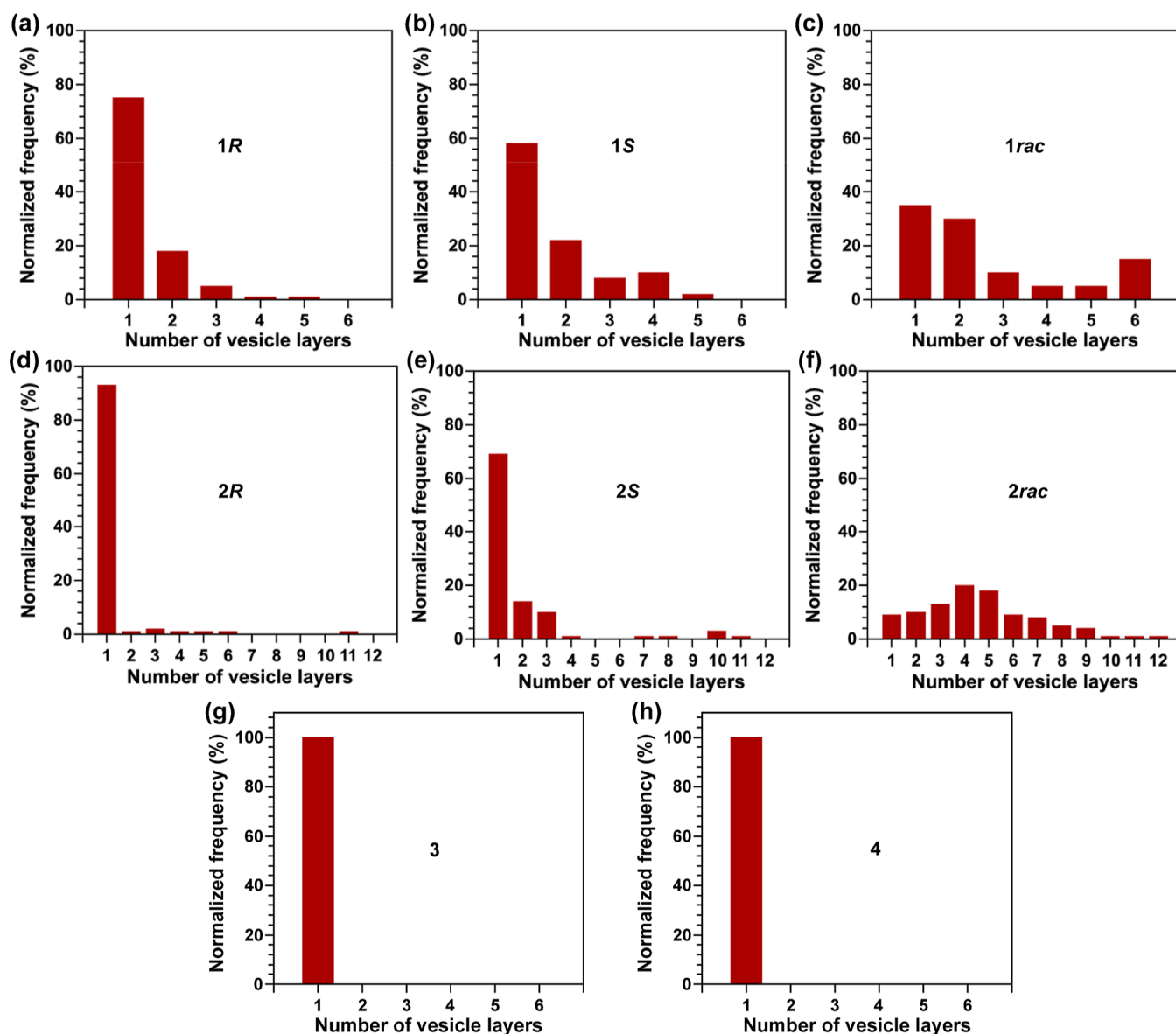


Figure 7. Histograms of the normalized frequency of number of vesicle layers of DSs assembled from glycerol JDs **1R** (a), **1S** (b), **1rac** (c), **2R** (d), **2S** (e), and **2rac** (f) and achiral JDs **3** (g) and **4** (h) by statistical analysis. Number of DSs counted for each glycerol JD: 82 (**1R**), 40 (**1S**), 20 (**1rac**), 216 (**2R**), 92 (**2S**), 306 (**2rac**), 10 (**3**), and 3 (**4**).

aggregate (approximately 35 nm). In addition, we characterized the target JD membrane by calculating the typical membrane properties, area per lipid, line tension, and bending modulus using the built CG model. The calculated membrane properties are listed in Table S8.

For the JD **2rac** system, the aggregate formed a more compact, onion-like structure compared to the other systems. While the JD **2R**, **3**, and **4** aggregates formed swelling regions on the outermost layer, the JD **2rac** aggregate did not form such regions and included stacked layers. These results agree well with the experimental result, which indicate that **2rac** JDs have the potential to form onion-like structures in comparison to other chiral and achiral JDs, although the other JDs could not attain complete unilamellar vesicles within the simulation time scale (even though the physical time scale of CG MD can be 10 times longer than the simulation time; which means effectively 20 μ s). Although the physical membrane properties of the JD aggregates have been explored, no significant

uniqueness of the **2rac** bilayer membrane was detected (Table S8). However, the line tensions of the achiral JDs are higher than those of the chiral JDs, which indicates that the bilayer structure of achiral JD aggregates is more stable than that of chiral JDs. This is because a high line tension can prevent water leakage through vesicular layers. This finding may also correspond to the experimental result that the achiral JDs can maintain and only form unilamellar vesicles, which are stable without multilamellar stacking.

Simulation times on the order of microseconds may not have been sufficient to relax the aggregate structure. In fact, some hydrophilic pores remained on the aggregate surface even after our simulations conducted at relatively high temperatures with glycerol JDs **2R**, **3**, and **4**. Therefore, the aggregates in these systems may continue to grow as the simulation time is extended. The initial configuration of the simulated systems might also have affected the results obtained from the JD aggregate simulations. To investigate the stability

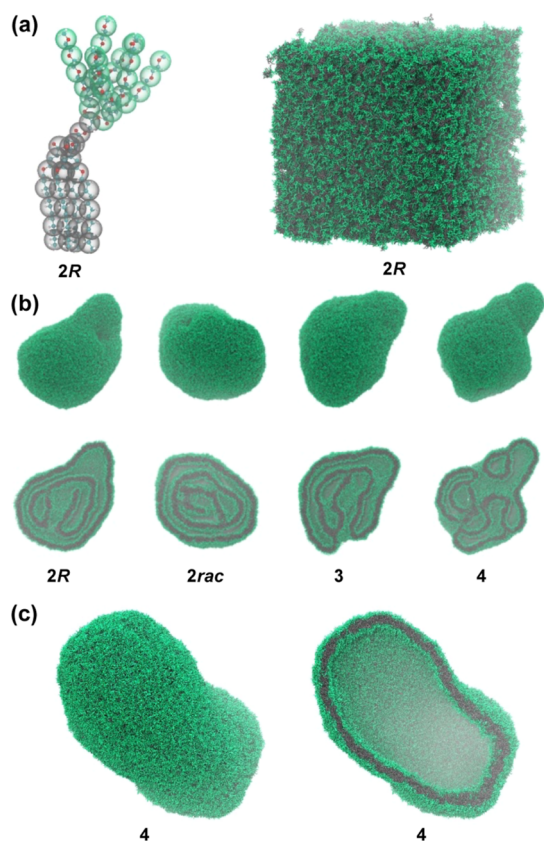


Figure 8. Simulations of DSs assembled from glycerol JDs **2R**, **2rac**, **3**, and **4**. (a) Left: coarse-grained **2R** JD structure. Right: initial configuration of the JD aggregate systems. Glycerol JD headgroups and tails are colored green and gray, respectively. Water is omitted for clarity. (b) Entire and cross-sectional views of the final configuration of microsecond-order CG MD for JD aggregates. (c) Entire (left) and cross-sectional (right) views of the final aggregate configuration in the achiral JD **4**.

of the unilamellar structure in the JD aggregate, we additionally carried out 500 ns CG MD analysis on the achiral JD **4** aggregate, through which its complicated internal structure was removed from the initial configuration provided by the previous simulation. Figure 8c shows the final configuration of the simulation. The observed unilamellar vesicle of symmetric achiral JD **4** was stable during the simulation. Pores that were presented in the initial configuration were closed quickly, and no significant morphology change of the vesicle was detected later. Thus, the CG MD simulations successfully demonstrate the apparent onion-like structure of **2rac**, clear difference in the line tension between chiral and achiral JDs, and stable unilamellar structure of the JD **4** assembly, which supports the DLS and cryo-TEM experimental outcomes.

CONCLUSIONS

Glycerol JDs with nonsymmetric homochiral, symmetric achiral, and nonsymmetric racemic branching points were synthesized. They self-assemble into monodisperse DSs by simple injection of their ethanol solution into water. DLS measurements showed that the resulting DSs are concentration-size dependent with an identical trend regardless of achiral, homochiral, or racemic branching point. Therefore, DLS experiments alone cannot discriminate between the DSs

assembled from these glycerol JDs. However, cryo-TEM and statistical analysis showed that homochiral JDs form predominantly unilamellar, while racemic JDs favor multilamellar, onion-like DSs. Symmetric achiral JDs produce only unilamellar DSs. Statistical analysis of the number of bilayers showed that homochiral JDs self-assemble predominantly unilamellar, achiral only unilamellar, while racemic predominantly multilamellar, onion-like DSs. CG MD simulations displayed similar results with racemic JD **2rac** showing the potential to form onion-like structures and achiral JD **4** showing a stable unilamellar structure, although homochiral JD **2R** could not form complete unilamellar structures in the limited simulation time of microseconds. This study provides a rationale for the selection of homochirality of phospholipids by nature since single-bilayer membranes are required for the construction of biological cell membranes. However, it also demonstrates that symmetric achiral lipids, which were most probably available in prebiotic primitive cells, provide the most stable vesicles.¹⁶ While the amplified structure of JDs investigated here was never part of the prebiotic or contemporary biological membranes, the results reported here may provide new hypothesis for the field of biological membranes generated from phospholipids. The less stable homochiral contemporary membranes are stabilized by transmembrane proteins, cholesterol, and glycoconjugates and thus overcome the higher stability of the prebiotic achiral primitive cells. The work reported here is complementary to that on the origins of homochirality addressed with self-assembling dendritic dipeptides¹⁷ and via deracemization in the crystal state.¹⁸ The mechanisms explaining the formation of unilamellar DSs from homochiral and achiral JDs and the potential formation of giant unilamellar DSs by simple injection,^{16d} as well as the role of chirality in drug delivery including in the targeted delivery of mRNA,¹³ are under investigation. Our previous research on the discovery of the self-assembly of amphiphilic JDs into multilamellar onion-like DSs^{14a} and GDSs^{14d} inspired immediate development of models to explain the onion concept by dissipative particle dynamics methods.¹⁹ The development of a molecular model that explains the mechanism of deracemization¹⁸ vs onion formation of racemic JDs is in progress, and it will be reported in an independent publication.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.3c00389>.

Experimental methods, synthetic procedures with complete characterization data, additional cryo-TEM data, statistical analysis data, and supplemental references (PDF)

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Notes

The authors declare no competing financial interest.

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