Encapsulation and Diffusion of Water−Soluble Dendrimers in a Bicontinuous Cubic Phase

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Encapsulation and Diffusion of Water-Soluble Dendrimers in a Bicontinuous Cubic Phase

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Bicontinuous cubic (Q_{II}) phases composed of hydrated lipids are a unique mesoporous organic material. The diameter of the water channels in the range of 5−10 nm could be used as a reservoir for macromolecular therapeutic agents. Here, we describe the synthesis of water-soluble poly(amidoamine) (PAMAM) dendrimer derivatives labeled with fluorine and their diffusion in the water channels of a Q_{II} phase. A Q_{II} phase with *Ia*3*d* symmetry was prepared by hydration of a 9:1 molar mixture of polymerizable monoacylglycerol and the corresponding 1,2-diacylglycerol. The dendrimers having fluorine were synthesized by Michael reaction of PAMAM dendrimers with a mixture of ethyl 4,4,4-trifluorocrotonate and methyl acrylate. The hydrated diameter of the fluorinated dendrimer of generation 2.5 (G2.5AFH) is 32.6 Å as measured by ^{19}F NMR. The diffusion coefficient of G2.5AFH at 25 °C in the water channels of the Q_{II} phase, determined by pulsed field gradient ^{19}F NMR spectroscopy, is 1×10^{-12} m^2/s, which is compared to the free diffusion coefficient of the dendrimer in water (1.42×10^{-10} m^2/s). These data indicate that small globular proteins or similar molecules can diffuse rapidly enough in stabilized Q_{II} phases to be technically useful.

Hydrated amphiphiles exhibit various lamellar and nonlamellar assemblies depending on concentration, temperature, and pressure.[1−3] The hydrated lipids yield complex lyotropic liquid crystals, that is, monolayers, bilayers, inverted hexagonal (H_{II}), and bicontinuous cubic (Q_{II}) phases. Such morphologies have also been observed for amphiphilic block copolymers.[4,5] A number of Q_{II} phases can exist between lamellar and the H_{II} phase. The Q_{II} phases are bicontinuous with respect to the water channels and the curved lipid regions and are considered the organic analogues of zeolites. The large water channels of Q_{II} phases (5−10 nm in diameter), compared to that of the H_{II} phase (about 2 nm in diameter), suggest the possibility that they could be used as a reservoir for relatively large molecular drugs or proteins.

To increase the potential use of the lipid assemblies, the nonlamellar architectures have been polymerized and their temperature and concentration ranges are extended.[6−8] A 3:1 molar mixture of a mono-dienoylphosphatidylethanolamine (mono-DenPE) and bis-dienoylphosphatidylcholine (bis-DenPC) formed a Q_{II} phase of *Pn*3*m* space group at temperatures greater than 55 °C.[7] Following polymerization, the cross-linked Q_{II} phase could exist at temperatures as low as ca. 0 °C. Recently, it was also reported that hydration of a 9:1 molar mixture of polymerizable monoacylglycerol and the corresponding diacylglycerol resulted in a Q_{II} phase with *Ia*3*d* symmetry.[8] The diameter of the water channels was estimated to be about 8 nm. Polymerization of this isotropic Q_{II} phase was shown to expand the upper boundary temperature from 45 to at least 70 °C.[8] Because Q_{II} phases may undergo phase transformation by incorporation of additional components, polymerization is also expected to contribute to their dimensional stability.

Studies of the release of encapsulated drugs or proteins in Q_{II} phases suggest that the release occurs by diffusion in the water channels,[9−11] but factors such as charge, shape, and size of the solutes as well as pH and ionic strength of the medium should also influence the release behavior. For an initial evaluation of the effect of molecular size on the diffusion of water-soluble macromolecules in a Q_{II} phase, we selected dendrimers. The various generations of poly(amidoamine) (PAMAM) dendrimers are water-soluble and have a nearly spherical shape of defined sizes;[12−14] therefore, they can serve as useful model compounds for globular proteins. In this study of diffusion of macromolecules in a Q_{II} phase, the PAMAM dendrimers

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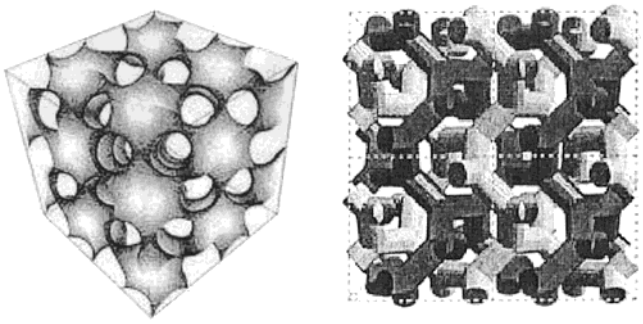
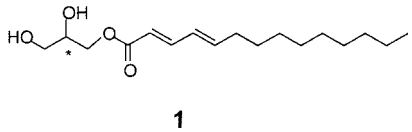


Figure 1. Mathematically generated minimal surfaces of a bicontinuous cubic phase of lipids with *Ia*3*d* symmetry and its labyrinth that represents the three-dimensional networks of the water portion of the phase obtained from hydrated lipids (ref 5).



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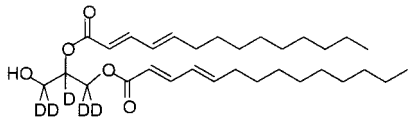

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Figure 2. Polymerizable monoacylglycerol (**1**) and 1,2-diacylglycerol (**2**).

were labeled with fluorine in order to observe the fluorine signal during pulsed field gradient ^{19}F NMR.

In this paper, we describe the preparation of fluorine-containing dendrimers and their diffusion in the water channels of a Q_{II} phase (see Figure 1). It is already well-known that the membrane surface of lipid layers can be modified by incorporation of functionalized lipids.[15] Consequently, functionalization of the water channel surface of Q_{II} phases is expected to offer many applications in biological and material sciences (e.g., catalysis, separation, drug delivery, and sensors, among others).

Experimental Section

Syntheses. Chemical reagents and starting materials were purchased from Aldrich Chemical Co., unless otherwise noted. Chloroform and dichloromethane were distilled from calcium hydride prior to use. Dimethylformamide (DMF) was dried over 3 Å molecular sieves. All other reagents were used as received without further purification. All reactions were performed under an argon atmosphere unless indicated otherwise. The ^{1}H and ^{13}C NMR spectra were recorded using a 200 MHz spectrometer. Compounds containing UV-sensitive groups were handled under yellow light. The reactions were monitored by thin-layer chromatography (TLC) that was visualized by an UV lamp, iodine vapor, or staining with phosphomolybdic acid (5% in ethanol). Mass spectroscopy was performed by the mass spectroscopy facility at the University of Arizona. 1-[2,4-(*E*,*E*)-Tetradecadienoyl]-*sn*-glycerol (**1**) (see Figure 2) was prepared according to a known procedure.[16]

*3-(4-Methoxybenzyl)-rac-glycerol-d$_5$ (**5**).* A solution of 1,2-*O*-isoproylidene-*rac*-glycerol-*d*$_5$[16] (**3**) (244 mg, 1.78 mmol) in DMF (2 mL) was added dropwise to a suspension of NaH (107 mg, 60% dispersion in mineral oil, 2.67 mmol) in DMF (10 mL). The reaction mixture was stirred for 30 min, and 4-methoxybenzyl chloride (335 mg, 2.14 mmol) was added. The reaction was followed by TLC using hexane/ethyl acetate (4:1) as the mobile phase. After compound **3** was totally consumed, water was added slowly to quench the excess NaH. The reaction mixture was diluted with ether (100 mL) and extracted with water (20 mL × 5) and brine (20 mL). The organic layers were dried over $MgSO_4$ and concentrated. The crude product was purified by flash column chromatography using hexane/ethyl acetate (4:1) as the eluent to give 401 mg of 1,2-*O*-isoproylidene-3-(4-methoxybenzyl)-*rac*-glycerol-*d*$_5$ (**4**) (88% yield): R_f = 0.43 in hexane/ethyl acetate (4:1); ^{1}H NMR ($CDCl_3$) δ 7.24−7.28 (d, J = 8.6 Hz, 2H), 6.86−6.90 (d, J = 8.6 Hz, 2H), 4.50−4.51 (d, J = 1.9 Hz, 2H), 3.80 (s, 3H), 1.36 (s, 3H), 1.42 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 159.22, 130.00, 129.35, 113.74, 109.30, 73.06, 55.20, 26.74, 25.34; HRFAB (*m*/*z*) calcd for $C_{14}H_{15}O_4D_5$ 257.1675, found 257.1676.

To a solution of compound **4** (401 mg, 1.56 mmol) in methanol (5 mL) was added *p*-toluenesulfonic acid monohydrate (26 mg, 0.14 mmol). The reaction was followed by TLC using ethyl acetate as the mobile phase. After compound **4** was totally consumed, $NaHCO_3$ (24 mg) was added and the reaction mixture was concentrated. The residue was purified by flash column chromatography using hexane/ethyl acetate (1:3) as the eluent to give 258 mg of a white solid (76% yield): R_f = 0.38 in CH_2Cl_2/MeOH (95:5); ^{1}H NMR ($CDCl_3$) 7.22−7.26 (d, J = 8.6 Hz, 2H), 6.85−6.90 (d, J = 8.6 Hz, 2H), 4.46 (s, 2H), 3.79 (s, 3H), 3.12 (br, 1H), δ 2.76 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 159.27, 129.71, 129.40, 113.80, 73.06, 55.18; HRFAB (*m*/*z*) calcd for $C_{11}H_{11}O_4D_5$ 217.1362, found 217.1362.

*1,2-Bis[(E,E)-2,4-tetradecadienoyl]-rac-glycerol-d$_5$ (**2**).* A solution of compound **5** (258 mg, 1.19 mmol), (*E*,*E*)-2,4-tetradecadienoic acid[16] (532 mg, 2.37 mmol), and (dimethylamino)pyridine (145 mg, 1.19 mmol) in chloroform (20 mL) was cooled to 0 °C, and then *N*,*N*′-dicyclohexylcarbodiimide (734 mg, 3.56 mmol) was added. After the solution was stirred overnight at room temperature, the urea was filtered and the filtrate was concentrated. The concentrate was purified by flash column chromatography using hexane/ethyl acetate (9:1) as the eluent to give 1,2-bis[(*E*,*E*)-2,4-tetradecadienoyl]-3-(4-methoxybenzyl)-*rac*-glycerol-*d*$_5$ (**6**), contaminated with the corresponding *N*-acylurea. The crude product was dissolved in 10% trifluoroacetic acid in dichloromethane (10 mL), and the reaction mixture was stirred vigorously for 1 h at 0 °C. The reaction contents were concentrated in vacuo, and the crude product was purified by flash column chromatography using hexane/ethyl acetate (4:1) as the eluent to give 247 mg of a white solid (41% yield): R_f = 0.28 in hexane/ethyl acetate (4:1); ^{1}H NMR ($CDCl_3$) δ 7.32−7.22 (m, 2H), 6.20−6.17 (m, 4H), 5.83−5.74 (m, 4H), 2.31 (br, 1H), 2.18−2.12 (m, 4H), 1.41−1.19 (m, 28H), 0.87 (t, J = 6.37 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 167.35, 166.95, 146.52, 146.45, 146.22, 128.51, 118.67, 118.50, 33.40, 32.28, 29.92, 29.82, 29.69, 29.58, 29.07, 23.07, 14.27; HRFAB (*m*/*z*) calcd for $C_{31}H_{48}D_5O_5$ 510.4207, found 510.4204.

Synthesis of PAMAM G2.5F Dendrimer. To a stirred and cooled (ice/water) solution of ethyl 4,4,4-trifluorocrotonate (ETFC) (294 μL, 1.967 mmol) in anhydrous methanol (5 mL) was added a solution of PAMAM dendrimer of generation 2.0 (G2.0; 100 mg, 0.491 mequiv of NH_2) in methanol. After the ice/water bath was removed, the reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated in vacuo to give a dendrimer, denoted as G2.5F: ^{1}H NMR ($CDCl_3$) δ 8.10−7.20 (m), 4.17 (q, J = 7.0 Hz), 3.72 (s), 3.65−3.43 (m), 3.40−3.15 (m), 3.05−2.25 (m), 1.27 (t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) δ 172.68, 172.44, 170.42, 134.74, 129.10, 123.48, 117.84, 61.12, 56.79, 56.21, 52.39, 52.11, 50.12, 47.34, 39.51, 37.68, 37.49, 34.67, 34.01, 14.07; MALDI-TOF (*m*/*z*) 5947.5627.

Synthesis of G2.5AFH, G3.5AFH, and G4.5AFH. To a stirred and cooled (ice/water) solution of ETFC (294 μL, 1.967 mmol) and methyl acrylate (177 μL, 1.967 mmol) in anhydrous methanol (5 mL) was added a solution of PAMAM dendrimer of generations 2.0, 3.0, and 4.0 (G2.0, G3.0, and G4.0; 0.491 mequiv of NH_2) in methanol. After the ice/water bath was removed, the reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated in vacuo to give dendrimers, denoted as G2.5AF, G3.5AF, and G4.5AF, respectively: ^{1}H NMR ($CDCl_3$) for G2.5AF δ 8.10−7.20 (m), 4.17 (q, J = 7.0 Hz), 3.67 (s), 3.63 (s), 3.62−3.43 (m), 3.40−3.15 (m), 3.05−2.25 (m), 1.27 (t, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$) for G2.5AF δ 173.0, 172.5, 172.3, 61.1, 56.9, 52.9, 52.4, 51.6, 50.0, 49.3, 39.6, 37.5, 37.2, 34.7, 34.0, 33.8, 32.7, 17.2.

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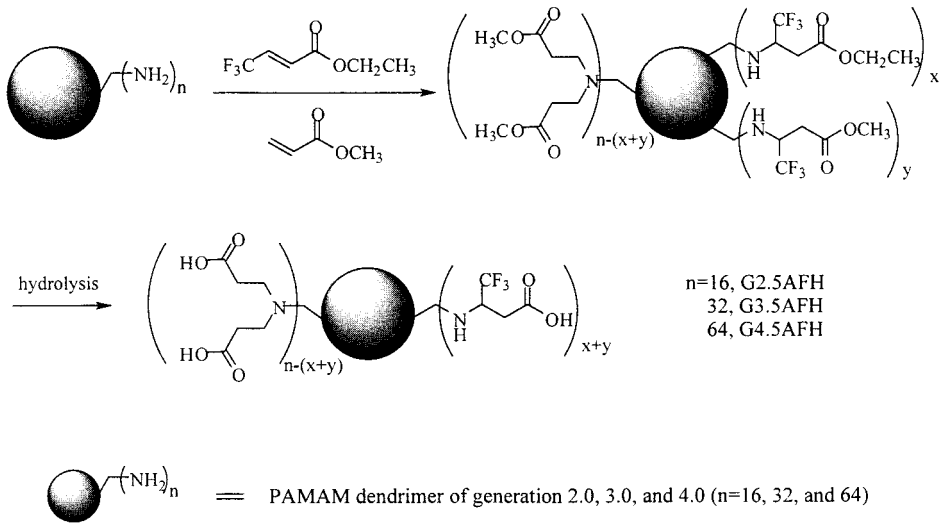

Figure 3. Preparation of the fluorine-labeled PAMAM dendrimers.

A 50 wt % NaOH solution (52 μL, 0.98 mmol) was added to a stirred and cooled (ice/water) solution of G2.5AF, G3.5AF, and G4.5AF in methanol (3 mL) and water (1 mL). After the ice/water bath was removed, the reaction mixture was stirred overnight at room temperature. NH_4Cl (53 mg, 0.98 mmol) was added, and the reaction mixture was evaporated in vacuo. The remaining oil was treated with toluene and again evaporated in vacuo to give dendrimers, denoted as G2.5AFH, G3.5AFH, and G4.5AFH, respectively: ^{1}H NMR (D_2O) for G2.5AFH δ 3.52−3.40 (m), 3.40−2.95 (m), 2.85−2.20 (m); ^{13}C NMR ($CDCl_3$) for G2.5AFH δ 177.9, 177.8, 173.0, 172.3, 56.7, 52.0, 51.6, 50.8, 49.4, 46.2, 38.9, 36.5, 34.6, 30.4, 29.8, 29.4.

Pulsed Field Gradient NMR Spectroscopy. A Q_{II} phase encapsulating fluorine-containing dendrimers was prepared using the same method previously reported.[8] To a 9:1 molar mixture of **1** and **2**, 3% w/w aqueous solution of the dendrimer was added at the concentration of 25% water by weight. The sample was centrifuged and incubated at room temperature for a week. ^{19}F and ^{1}H pulsed field gradient NMR diffusion measurements were performed on a Varian CMX Infinity NMR spectrometer, operating at a proton frequency of 400 MHz, using the stimulated spin−echo pulse sequence.[17] The spectrometer was equipped with a double resonance diffusion probe capable of producing linear gradients up to 3.4 T/m. The 90° pulse lengths were 7 μs, and the waiting time between experiments was typically 3−5 times longer than the longitudinal relaxation time, T_1. The temperature was controlled to ±0.5 °C by means of a heated air stream passing outside the glass vessel containing the sample. The amplitude of the peaks was attenuated according to the Stejskal−Tanner equation:

$$A = A_0 e^{-\gamma^2 g^2 \delta^2 D(\Delta - \delta/3)} \quad (1)$$

where A and A_0 are the peak amplitudes with and without applied magnetic field gradients, γ is the magnetogyric ratio, g is the gradient strength, δ is the gradient pulse width, and Δ is the diffusion time.[17] Typically, 20 experiments were performed for each diffusion measurement, during which the gradient strength was varied and all the other parameters were kept constant, allowing for the peak amplitude to be fitted according to eq 1. Several such diffusion measurements were made on each sample with Δ varying between 10 and 200 ms (^{19}F) or between 6 and 1000 ms (^{1}H). No variation of D on Δ was observed.

Results and Discussion

Synthesis of Lipid 2. Lipid **2** was prepared according to a known procedure with some modification.[16] Reaction of 3-(4-methoxybenzyl)-*rac*-glycerol-d_5 (**5**) with (*E*,*E*)-2,4-tetradecadienoic acid in the presence of *N*,*N*′-dicyclohexylcarbodiimide and (dimethylamino)pyridine yielded the 4-methoxybenzyl ether (PMB)-protected 1,2-diacylglycerol (**6**) along with the corresponding *N*-acylurea. A slow reaction at the C2 position of compound **5** presumably facilitated the production of the *N*-acylurea. Compound **6** which was contaminated with the *N*-acylurea after column chromatography was used for the next deprotection reaction without further purification. Trifluoroacetic acid (TFA) has been used for deprotection of the PMB group.[18,19] Treatment of the crude **6** with 10% TFA in dichloromethane at 0 °C for 1 h gave lipid **2** cleanly without affecting the *N*-acylurea. In the deprotection reaction of the PMB group, TFA is comparable to dimethylboron bromide.[16] Lipid **2** was obtained in 41% yield (acylation and deprotection) after flash column chromatography. There was no detectable isomerization of 1,2-diacylglycerol to 1,3-diacylglycerol as shown by NMR and TLC of the isolated product in the solvent system of toluene, chloroform, and methanol (85:15:5).

Synthesis of Fluorine-Containing Dendrimers. To investigate the size-dependent diffusion of macromolecules in the water channels of a Q_{II} phase by ^{19}F NMR spectroscopy, fluorine was introduced into PAMAM dendrimers of generations 2.0, 3.0, and 4.0 (G2.0, G3.0, and G4.0) (Figure 3). G2.0 was reacted with 4 equiv of ETFC to the amine groups of the dendrimer in anhydrous methanol for 2 days at room temperature (G2.5F). In the ^{1}H and ^{13}C NMR spectra of G2.5F, the disappearance of vinyl group signals indicates that ETFC has reacted with the amines by Michael addition rather than amidation. The appearance of the methoxy group signal may be a result of the partial transesterification of the dendrimer ethyl ester by methanol solvent. In the MALDI-MS spectrum, the peak at *m*/*z* 5947.5627 corresponds to the average molecular weight of G2.0 plus 16 ETFC. A series of peaks with the difference of a mass of 14 u following the peak at *m*/*z* 5947.5627 also show the partial exchange of the dendrimer ester. About 10% of ethyl ester was converted into methyl ester. The next mass signals appear from the peak at *m*/*z* 5779.3777 with the difference of a mass of 14 u, which can be assigned to the dendrimer G2.0 that reacted with 15 ETFC molecules. The peaks from *m*/*z* 5665.4036 with the difference of a mass of 14 u seem to arise from a structure defect of starting G2.0 which lacks one $-CH_2CHCONHCH_2CH_2NH_2$ unit (G2.0′ + 15

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Table 1. Characterization of Fluorine-Containing Dendrimers

generation	average no. of ETFC in one dendrimer molecule[a]	mass (*m*/*z*) (ester)[b]	mass (*m*/*z*) (carboxylate)[b]
2.5	4.1	5893	5476
3.5	10.2	11801	11260
4.5	18.9	23420	21475

[a] Calculated from the ratio of OCH_2CH_3 (1.27 ppm) to $CONHCH_2CH_2$ (3.40−3.15 ppm) by ^{1}H NMR. A 10% exchange of ethoxy groups of the pendant ETFC with methoxy groups is assumed. [b] The most intense mass signal in the MALDI-MS.

ETFC). Under the reaction conditions, each amine group of G2.0 reacted with only one ETFC, while it reacted with two methyl acrylate (Figure 3). After further reaction of G2.5F with excess methyl acrylate (4 equiv), a new peak at *m*/*z* 5950.3728 appeared and the peaks at *m*/*z* 5779.3777 disappeared in the MALDI spectrum. The one remaining free amine group of the dendrimer that has reacted with 15 ETFC reacted further with two methyl acrylate.

The fluorine-containing dendrimers with more terminal carboxylate groups (G2.5AF, G3.5AF, and G4.5AF) were prepared by the reaction of PAMAM dendrimers (G2.0, G3.0, and G4.0) with a mixture of 4 equiv of ETFC and 4 equiv of methyl acrylate to the amines of the dendrimers, respectively. ^{1}H NMR spectra of the dendrimers show that 26−32% of the PAMAM dendrimer amines reacted with ETFC (Table 1). The mass signals distributed between *m*/*z* 5500 and *m*/*z* 6000 were shown in the MALDI spectrum of G2.5AF, indicating some structure defects of the dendrimer. The most intense mass signal was the peak at *m*/*z* 5893. The MALDI spectra of G3.5AF and G4.5AF also show a distribution around the most intense peak similar to that of G2.5AF. The resulting dendrimers were hydrolyzed by use of NaOH in a mixture of methanol and water to give the carboxylate-functionalized dendrimers. Complete hydrolysis was confirmed by the ^{1}H and ^{13}C NMR spectra.

Diffusion of Dendrimers. First, the diffusion coefficient of the dendrimer was determined in water. The fluorine diffusion coefficient of a 3% w/w aqueous solution of G2.5AFH at 25 °C was found to be 1.42×10^{-10} m^2/s. Using the Stokes−Einstein equation, the diffusion coefficient of G2.5AFH extrapolated to infinite dilution results in a hydrated diameter of 32.6 Å for the compound.[20,21] This value is reasonable, compared with the diameter of similar dendrimer compounds obtained from size exclusion chromatography and molecular simulations or neutron scattering.[13,14]

The cubic phase that encapsulated the fluorine-containing dendrimers was prepared by addition of 3% w/w aqueous solution of the dendrimers to a 9:1 molar mixture of **1** and **2** at the concentration of 25% water by weight, followed by centrifugation and incubation at room temperature for a week.[8] The obtained samples were not completely optically isotropic. Instead, typically some of the volume of the 8 mm thick sample showed a weak birefringence when viewed between crossed polarizers, which suggests that a small portion of the sample had not become isotropic. There were also indications of small crystallites in some of the samples, visible both by the eye and by polarized optical microscopy. A portion of the ^{19}F NMR signal was broad, typically 6−7 kHz, also indicating the presence of an anisotropic phase, as well as the isotropic cubic phase. However, a portion of the signal was sufficiently narrow to produce an echo in the pulsed field gradient NMR experiment. The diffusion coefficient of this component was ca. 1×10^{-12} m^2/s for G2.5AFH and slightly lower for the other two dendrimers (G3.5AFH and G4.5AFH) studied. The possibility of unencapsulated dendrimer was excluded by the absence of a faster diffusing component. The lipid diffusion was also measured in the sample containing G2.5AFH, and it was found to be 5.5×10^{-12} m^2/s. This value is in the range found for lipids in bicontinuous cubic phases.[22] Finally, a Q_{II} phase sample composed of the classic monooleoylglycerol (monoolein) and a 3% w/w G2.5AFH aqueous solution showed the same diffusion behavior as determined for samples from lipids **1** and **2**.

Conclusions

The water-soluble PAMAM dendrimers labeled with fluorine were prepared by the Michael addition of amine-functionalized PAMAM dendrimers to a mixture of ETFC and methyl methacrylate, followed by basic hydrolysis. The fluorinated dendrimer size was obtained from the fluorine diffusion coefficient in an aqueous solution. The hydrated diameter of the dendrimer of generation 2.5 (G2.5AFH) is 32.6 Å, which corresponds to the average diameter of PAMAM dendrimers of generations 2.0 and 3.0. The diffusion coefficient of G2.5AFH in the water channels of a Q_{II} phase, determined by pulsed field gradient NMR experiments, is 1×10^{-12} m^2/s, 2 orders of magnitude smaller than the free diffusion coefficient of the dendrimer in water. The magnitude of this diffusion coefficient is most likely due to the tortuous path of the water channel in the Q_{II} phase as well as hydrogen-bonding interactions of the G2.5AFH surface carboxylates with the lipid primary hydroxyl group at the lipid−water interface. A determination of the relative importance of these effects must await further studies. These data provide the first estimate of the diffusion of globular macromolecules in Q_{II} phases. The results suggest that small globular proteins or similar molecules could be encapsulated in stabilized biocompatible and mesoporous Q_{II} phases and diffuse rapidly enough to be technically useful.

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