

## Films of Agarose Enable Rapid Formation of Giant Liposomes in Solutions of Physiologic Ionic Strength

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**Abstract:** This paper describes a method to form giant liposomes in solutions of physiologic ionic strength, such as phosphate buffered saline (PBS) or 150 mM KCl. Formation of these cell-sized liposomes proceeded from hybrid films of partially dried agarose and lipids. Hydrating the films of agarose and lipids in aqueous salt solutions resulted in swelling and partial dissolution of the hybrid films and in concomitant rapid formation of giant liposomes in high yield. This method did not require the presence of an electric field or specialized lipids; it generated giant liposomes from pure phosphatidylcholine lipids or from lipid mixtures that contained cholesterol or negatively charged lipids. Hybrid films of agarose and lipids even enabled the formation of giant liposomes in PBS from lipid compositions that are typically problematic for liposome formation, such as pure phosphatidylserine, pure phosphatidylglycerol, and asolectin. This paper discusses biophysical aspects of the formation of giant liposomes from hybrid films of agarose and lipids in comparison to established methods and shows that gentle hydration of hybrid films of agarose and lipids is a simple, rapid, and reproducible procedure to generate giant liposomes of various lipid compositions in solutions of physiologic ionic strength without the need for specialized equipment.

### Introduction

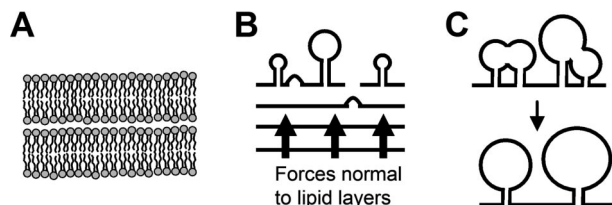
Giant liposomes are useful models to study cellular membranes since they approximate the size and membrane curvature of live cells<sup>1–6</sup> and since they can be observed individually by optical microscopy.<sup>2,3,5–7</sup> Several methods produce giant liposomes; these methods include “gentle hydration”,<sup>7–11</sup> “freeze-and-thaw”,<sup>12,13</sup> “solid hydration”,<sup>14</sup> “solvent evaporation”,<sup>15,16</sup> emulsion-based methods,<sup>17</sup> and “electroformation”.<sup>1,2,9,18–21</sup>

These methods generate giant liposomes of high quality and yield, but they are typically limited to solutions of low ionic strength ( $\leq 50$  mM monovalent salt)<sup>1,3,7–10,16,22</sup> unless specialized lipid formulations<sup>8–10</sup> or specific electroformation protocols<sup>22,23</sup> are used.

Due to this limitation, most biophysical and biochemical studies on giant liposomes so far have been carried out in aqueous solutions with an ionic strength significantly below the physiologic range.<sup>22,23</sup> At least six important properties of giant liposomes can, however, be affected by ionic strength; these include: (i) electrostatic interactions of lipid membranes with proteins,<sup>24–26</sup> with adjacent lipid membranes,<sup>22,27</sup> or with other molecules or ions in solution;<sup>27–30</sup> (ii) osmotic properties;<sup>5,7,31,32</sup> (iii) packing of lipid headgroups in the membrane;<sup>5,33</sup> (iv) curvature,<sup>7</sup> bending elasticity,<sup>34</sup> and mechanical stability of lipid bilayers;<sup>5,35</sup> (v) activity of membrane proteins;<sup>36</sup> and (vi) ion

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**Figure 1.** Cartoon illustrating three important stages during the formation of giant liposomes. (A) Orientation and self-assembly of lipids into bilayers, leading to the formation of lipid lamellae.<sup>1,10,11</sup> (B) Growth of liposomes promoted by forces normal to the bilayers.<sup>3,42</sup> (C) Fusion of adjacent liposomes into giant liposomes due to crowding and associated mechanical forces.<sup>2,41,46</sup> The black lines in B and C represent lipid bilayers.

channel conductance.<sup>35–37</sup> Consequently, it would be desirable to prepare giant liposomes in solutions that make it possible to extend these studies to physiologically relevant salt concentrations.<sup>22</sup>

To comprehend why it is difficult to form giant liposomes in solutions of physiologic ionic strength, a detailed understanding of the molecular mechanisms of formation of giant liposomes would be helpful. While the exact mechanisms of formation of giant liposomes still present questions,<sup>23,33,38–40</sup> the process can be separated into three stages as illustrated in Figure 1.<sup>1,3,10,41,42</sup> In the first stage of the formation of giant liposomes, lipids in a solid lipid film are hydrated, leading to the self-assembly and separation of lipid lamellae in the film.<sup>1,10,11</sup> This process can be promoted by electrostatic repulsion of negatively charged lipids,<sup>8,22,32,43</sup> steric effects from bulky headgroups,<sup>10,44</sup> and by prehydrating the lipid film<sup>8,10,11</sup> to orient lamellae of lipids and to separate the bilayers. Solutions of high ionic strength hinder this first step of separation of lamellae due to electrostatic screening of repulsive charges.<sup>22,27</sup> The second stage in the formation of giant liposomes involves swelling of liposomes due to forces normal to the bilayers<sup>3,42</sup> and recruitment of lipids from the lipid film.<sup>1,22</sup> Osmotic pressure,<sup>11,42</sup> line tension,<sup>42</sup> and electric fields<sup>42,45</sup> can provide this force, but can also be affected

by solutions of high ionic strength. The third and final stage involves fusion of adjacent liposomes due to mechanical stresses.<sup>33,41</sup>

Here we intended to promote all three stages of liposome formation in order to develop a method for the formation of giant liposomes in solutions of physiologic ionic strength, such as phosphate buffered saline (PBS), without the requirement for specialized lipids,<sup>8–10</sup> specialized equipment,<sup>23</sup> or a separate prehydration step.<sup>8–11</sup> We hypothesized that forming giant liposomes from a hybrid film of hydrogel and lipids may (i) promote the separation of lipid lamellae by providing prehydration and preorientation of lipids; (ii) promote growth of liposomes by generating forces normal to the lipid lamellae during the swelling of the agarose film; and (iii) promote fusion of adjacent liposomes due to crowding of growing liposomes that are attached to the swelling, porous film of agarose.

## Results and Discussion

Figure 2 illustrates the procedure of forming giant liposomes from hybrid films of agarose and lipids. This method proceeded in three simple steps and formed giant liposomes in physiological buffers within minutes after hydrating the hybrid films. The first step generated a film of ultralow melting agarose on a glass slide, the second step deposited lipids to generate a hybrid film of agarose and lipids, and the third step hydrated this film in solutions of physiologic ionic strength to generate giant liposomes.

To illustrate the benefit of forming giant liposomes from hybrid films of agarose and lipids in ionic solutions, we compared this method to the established method of “gentle hydration,” that is, the formation of liposomes from lipid films formed directly on bare glass substrates without agarose. Figure 3 shows that giant liposomes formed from films of agarose and lipids within a few minutes in remarkable yield in PBS solutions. Since we used optical phase-contrast and fluorescence microscopy to observe the formation of liposomes, we could only resolve liposomes with diameters  $\geq 1 \mu\text{m}$ . We observed the formation of such giant liposomes only on the surface of the hybrid film of agarose and lipids. Figure 4 shows, however, that lipids penetrated the entire agarose film, and therefore small liposomes ( $\ll 1 \mu\text{m}$ ) likely formed within the agarose network.

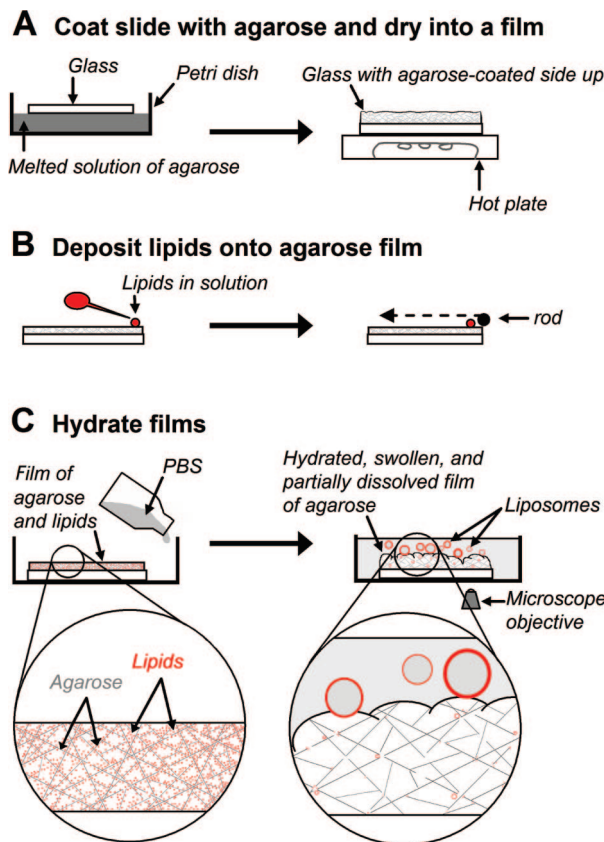
The giant liposomes that we could resolve on the surface of the hybrid film were typically surface-attached, appeared spherical (or polygonal when aggregated), and were often arranged in several layers above the surface.<sup>48,49</sup> Remarkably, giant liposomes formed over 30–80% of the surface of the chamber from a range of lipid compositions that included pure zwitterionic lipids (Figure 3A), lipids that typically pose difficulties for the formation of giant liposomes (such as pure DOPS, pure POPG, or asolectin; for full names of lipids, see figure caption of Figure 3)<sup>19</sup> (Figure 3C,E,G), as well as

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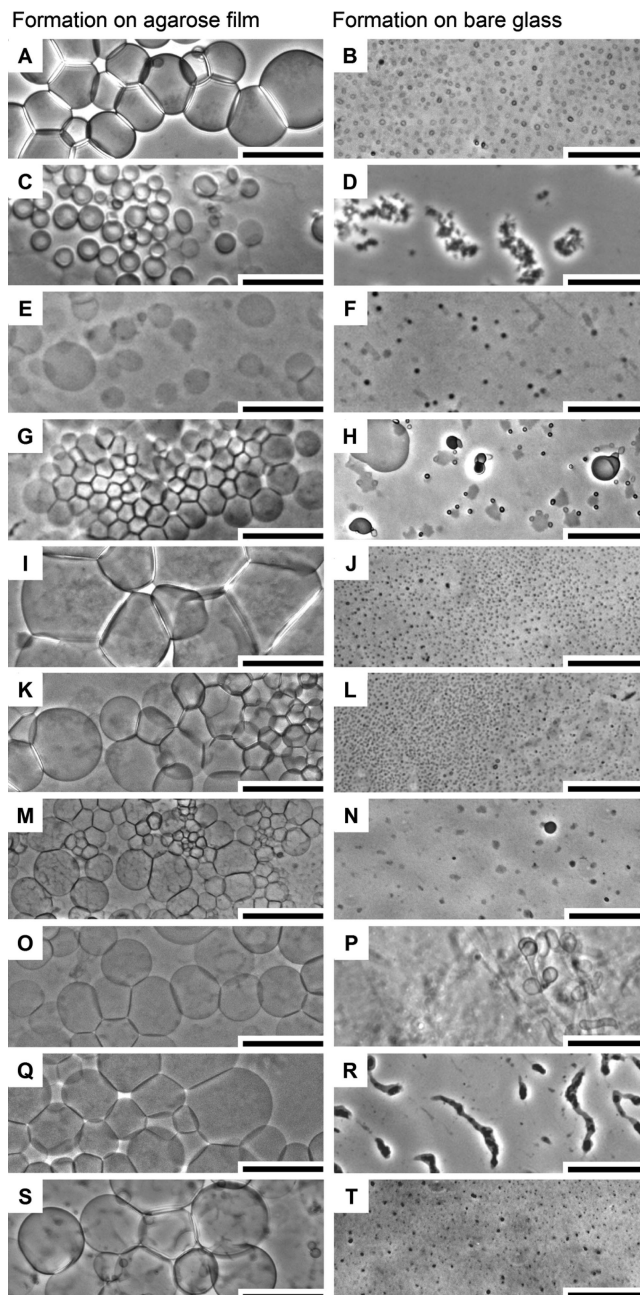
- (47) This trial was prepared on ITO plates instead of glass slides.
- (48) As new liposomes developed and swelled, previously formed liposomes were often pushed away from the surface. The liposomes in the resulting layers may be tethered to the surface or to other liposomes (some tethers were visible when viewed by phase contrast microscopy).
- (49) A few liposomes appeared to have detached completely but this occurrence was not common within any given population of liposomes. We procured free-floating liposomes by prying apart the chamber and allowing the contents to drip into a collection vessel or by applying gentle suction using a pipette or needle and syringe to remove the solution from the chamber (see Supporting Information, Figure S2).





**Figure 2.** Procedure of forming giant liposomes from hybrid films of agarose and lipids. (A) The procedure started by dipping one side of a clean glass slide in an aqueous solution of 1% (w/w) agarose with ultralow melting temperature (Type IX-A). After dripping off excess solution, the slide was turned and dried on a hot plate at a temperature of  $\sim 40^\circ\text{C}$  while keeping the agarose solution spread evenly over the glass slide (if necessary by moving a straight glass or metal rod tangentially over the surface during the drying process). This procedure generated a film of agarose with fairly uniform thickness on one side of the glass slide. (B) In the next step, a total volume of  $30\ \mu\text{L}$  of  $3.75\ \text{mg mL}^{-1}$  lipid in 90% (v/v) chloroform and 10% (v/v) methanol was spread evenly over (and into) the film of agarose by using a glass or metal rod while the solvents evaporated (see Supporting Information, Figure S1). To remove residual solvent, the glass slide with the resulting hybrid film of agarose and lipids was placed in a vacuum chamber (approximately  $-730\ \text{mTorr}$ ) for at least 20 min. (C) To generate liposomes, this slide was placed into a Petri dish such that the hybrid film of agarose and lipids faced upward and an aqueous solution containing 150 mM KCl, PBS, or deionized water was poured into the dish until it covered the slide.

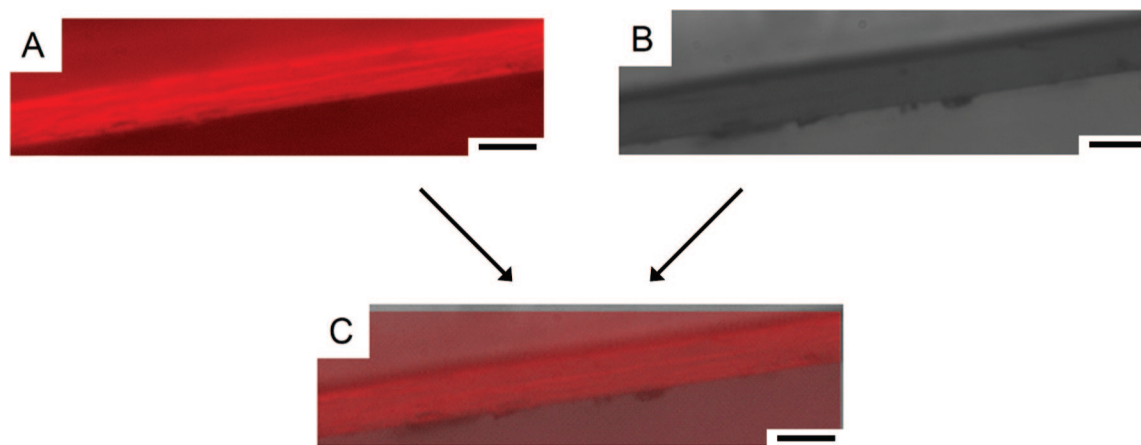
mixtures of zwitterionic lipids with 10 or 20 mol% cholesterol (Figure 3I,K), with 10 or 50 mol% negatively charged lipids (Figure 3M,O,Q), or with 5 mol% PEGylated lipids (Figure 3S). All these lipid mixtures formed hundreds of giant liposomes with diameters above  $10\ \mu\text{m}$  in solutions of physiologic ionic strength as well as in deionized water when grown from hybrid films of agarose and lipids. As expected,<sup>8,10</sup> we obtained the best liposomes with respect to their number, size, and surface coverage from lipid mixtures containing up to 50% negatively charged lipids or PEGylated lipids. The repulsive forces generated by these lipids also reduced the aggregation of liposomes that is typical in salt solutions.<sup>1,8–10,45,50</sup> With respect to the effect of the ions present in solution on the formation of giant liposomes, we found that giant liposomes formed best in



**Figure 3.** Phase contrast images of giant liposomes formed within 1 h in phosphate buffered saline (PBS). The column of images on the left shows liposomes that formed from hybrid films of agarose and lipids; the column on the right shows the control experiments of liposomes that formed from lipid films on bare glass. The following lipid compositions were used (in mol%): (A, B) Pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC). (C, D) Pure 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS). (E, F) Pure 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG). (G, H) Asolectin from soybean. (I, J) Mixture of 90% POPC with 10% cholesterol. (K, L) Mixture of 80% POPC with 20% cholesterol. (M, N) Mixture of 90% POPC with 10% of the negatively charged lipid POPG.<sup>47</sup> (O, P) Mixture of 50% POPC with 50% POPG. (Q, R) Mixture of 90% POPC with 10% of the negatively charged lipid DOPS. (S, T) Mixture of 95% POPC with 5% 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (also referred to as PEG-PE or PEGylated lipid). Scale bars =  $100\ \mu\text{m}$ .

deionized water, followed by 150 mM KCl (see Supporting Information, Figure S3) and PBS. In summary, formation of giant liposomes in PBS proceeded in high yield from all lipid compositions that we tried, as shown in Figure 3.

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**Figure 4.** Cross-section through a thick film of agarose that was coated with fluorescently labeled lipids. (A) Epifluorescence image of a cross-section of the film. (B) Phase contrast image of the cross-section of the film. (C) Overlay of images A and B with A at 50% transparency, indicating that the fluorescently labeled lipids penetrated completely through the film of agarose. Scale bars = 20  $\mu\text{m}$ .

Figure 3 illustrates that the presence of a film of agarose was essential for the formation of giant liposomes in solutions of physiologic ionic strength. Control experiments with lipid films deposited directly on glass or on surfaces of indium tin oxide (ITO), showed either no formation or only sporadic formation of giant liposomes (Figure 3B,D,F,H,J,L,N,P,R,T). The few giant liposomes that did form from lipid films on bare glass (or bare ITO) surfaces were typically smaller than 5  $\mu\text{m}$ . Interestingly, even lipid mixtures containing up to 50% negatively charged lipids or PEGylated lipids generated a very low yield of giant liposomes in PBS when formed from lipid films on bare glass surfaces (Figure 3N,P,R,T).<sup>51</sup>

To investigate if prehydration of the lipid film would improve the yield of giant liposomes from bare glass surfaces in PBS, we performed the protocol described by Akashi et al. using a lipid mixture containing 90% POPC and 10% POPG.<sup>8</sup> Using Akashi's method on bare glass surfaces with prehydration, we observed the formation of only a few giant liposomes in PBS; these liposomes were typically smaller than 10  $\mu\text{m}$ . These results are consistent with those by Akashi et al. who reported no formation of giant liposomes in solutions with KCl or NaCl concentrations greater than 100 mM.<sup>8</sup> Figure 3 hence illustrates that formation of giant liposomes from hybrid films of agarose and lipids provides a significant and important benefit over currently existing methods of formation of giant liposomes in solutions of physiologic ionic strength.

Before discussing the role of films of agarose in promoting the formation of giant liposomes in ionic solutions, it is necessary to understand the location of the lipids relative to the agarose molecules in the hybrid films of agarose and lipids prior to adding aqueous solution (i.e., at the end of step B in Figure 2). This aspect is important because the effect of interactions between lipid and agarose molecules on the formation of liposomes likely depends on the contact area between agarose molecules and lipid molecules. If lipids penetrated into the agarose film, then surface interactions

between lipids and agarose molecules in a porous agarose film would be increased due to the larger surface area compared to a flat surface (such as bare glass or the surface of a nonporous film). To test if lipids penetrated the film of agarose, we compared micrographs of particularly thick ( $\sim 16 \mu\text{m}$ ), hybrid films of agarose and fluorescently labeled lipids. Cross-sectional views of these films revealed that the fluorescently labeled lipids penetrated the entire thickness of the film of agarose (Figure 4).<sup>52</sup> Hence, the resulting hybrid film of agarose and lipids inevitably generated a large contact area between agarose and lipid molecules, and this extended interface area influenced the formation of lipid lamellae and giant liposomes as discussed below.

Another important factor for understanding how the hybrid film of agarose and lipids promoted the formation of giant liposomes is whether or not the film of agarose dissolved to some extent to form agarose molecules in solution. Agarose is a polysaccharide consisting of alternating residues of  $\beta$ -1,3-linked D-galactose and  $\alpha$ -1,4-linked 3,6-anhydro- $\alpha$ -L-galactopyranose (see Figure 5A).<sup>53–59</sup> This linear polymer adopts a structure of left-handed helices<sup>53,54,58</sup> that are stabilized by weak hydrogen bonds and intramolecular hydrophobic interactions.<sup>57,59,60</sup> As solutions transition into a gel, agarose helices aggregate to form long fibers containing 10 to  $10^4$  molecules.<sup>53,54,59</sup> Figure 5C illustrates a SEM image of a film of agarose that formed a gel before drying the film (e.g., standard melting temperature agarose). This film displayed surface features that appeared to contain fibrillar structures. In contrast, SEM images of films of agarose that dried without noticeably forming a gel (e.g., ultralow melting agarose) did not show these structures (Figure 5D). In the experiments presented here, we typically formed films with a thickness of  $\sim 2 \mu\text{m}$  composed of

(51) This low yield may be due, in part, because we formed these liposomes without a prehydration step.

(52) To eliminate signal from the autofluorescence of agarose, we first viewed a film of agarose without lipids and adjusted the camera settings until we could no longer observe fluorescence from the agarose film. These settings were then used to view the agarose film with fluorescently labeled lipids; its fluorescence was significantly stronger and easily detectable.

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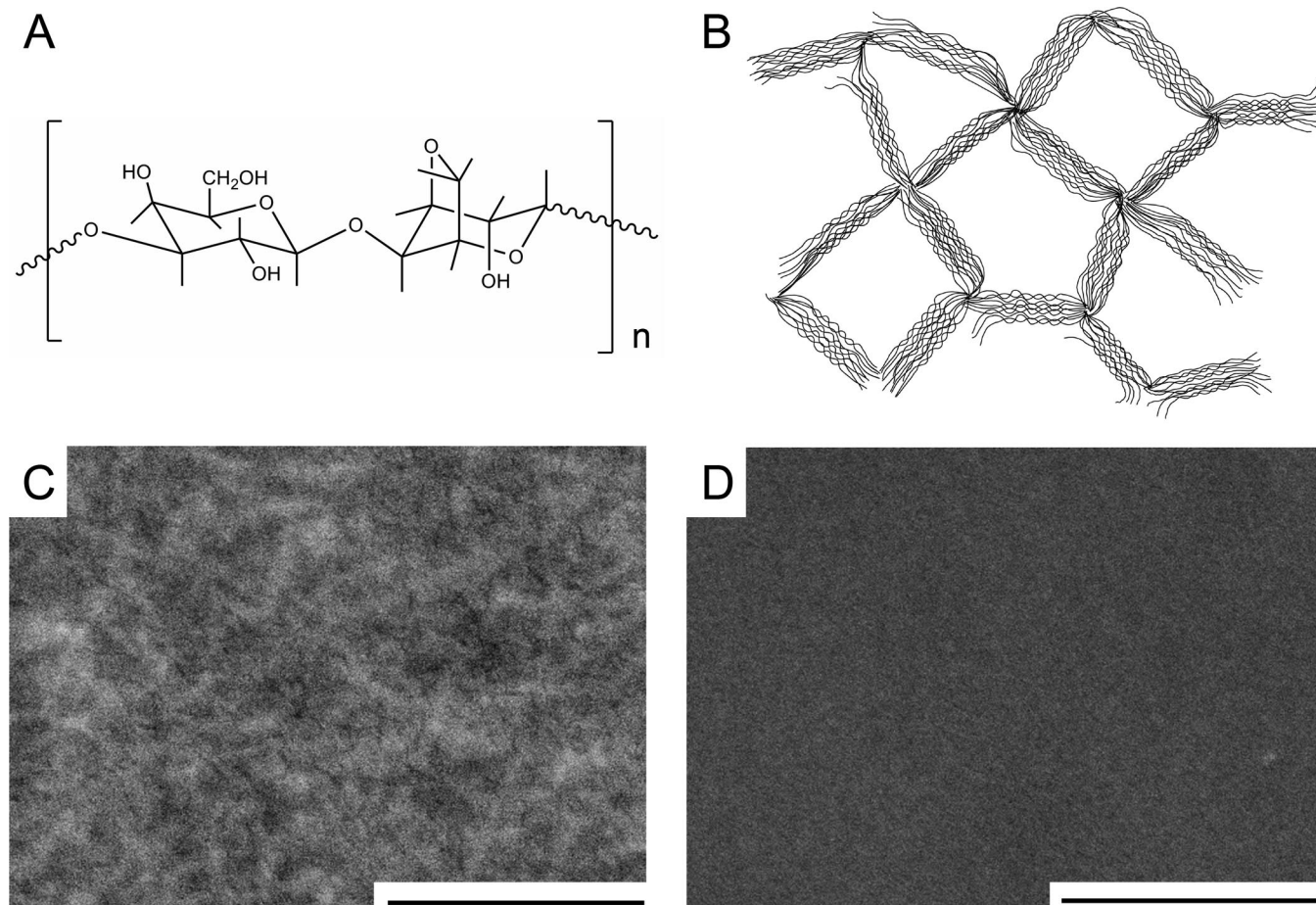
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**Figure 5.** Chemical and physical structure of agarose.<sup>53–55</sup> Agarose is a polysaccharide consisting of alternating residues of  $\beta$ -1,3-linked D-galactose and  $\alpha$ -1,4-linked 3,6-anhydro- $\alpha$ -L-galactopyranose, and the linear polymer forms left-handed helices.<sup>53,54,58</sup> (B) During formation of an agarose gel, 10 to  $10^4$  helices aggregate into long fibers,<sup>53,54,59</sup> which in turn associate in three dimensions to form a gel network.<sup>53,55–57</sup> Artwork adapted from Arnott et al.<sup>53</sup> (C) SEM micrograph of a film of standard melting temperature agarose. Note the appearance of fiber-like structures. (D) SEM micrograph of a film of ultralow melting agarose. No fiber-like structures could be resolved.<sup>61</sup> Scale bars = 400 nm.

ultralow melting temperature agarose by partially drying agarose solution on a glass surface. Foord and Atkins demonstrated by X-ray diffraction that drying a solution of agarose at elevated temperature resulted in a film that contained agarose molecules in the form of extended helices, which were not interlinked.<sup>54</sup> We therefore expected the agarose molecules in the films of ultralow melting agarose to be present in the form of extended helices that lay loosely over each other in random directions to form an unbound mesh of agarose molecules. Since the molecules were not tightly associated in a gel structure, it is likely that they can dissolve into solution. We demonstrate below that films of ultralow melting temperature agarose did, indeed, partially dissolve during liposome formation, and we discuss the possible importance of this characteristic for the formation of giant liposomes in solutions of physiologic ionic strength. We also show that agarose films that did not dissolve noticeably did not promote the formation of giant liposomes in PBS.

Since ultralow melting temperature agarose dissolves in solution, one important question is if agarose molecules associate with the membranes during the formation of liposomes. This question is relevant because binding of agarose molecules to liposomes may influence their formation in ionic solutions. To answer this question, we formed giant liposomes from a hybrid film of fluorescently labeled, ultralow melting temperature agarose and POPC lipids. Confocal microscopy revealed an increase in fluorescence intensity of giant liposomes formed

from films of fluorescently labeled agarose compared to the fluorescence intensity of liposomes formed from nonlabeled agarose (see Supporting Information, Figure S5). These results suggest that the agarose molecules associated with the liposome membranes. This association of macromolecular agarose molecules with lipid membranes may hence provide a similar benefit to the formation of giant liposomes as provided by PEGylated lipids.<sup>10</sup>

In order to determine the effect of membrane-associated agarose on the diffusivity of lipids in the membranes of liposomes formed from hybrid films of agarose and lipids, we performed fluorescence recovery after photobleaching (FRAP) experiments.<sup>29,62</sup> We found that the lipids in the resulting giant liposomes were mobile (see Supporting Information, Figure S6),

(61) We characterized the surface of films prepared from four types of agarose by AFM and SEM (see Supporting Information, Figure S4) and found that films from ultralow melting agarose appeared to display the smoothest (i.e., the least fibrous looking) surface. We attribute this observation to two reasons: (1) the average molecular weight of ultralow melting agarose was the lowest of the four types of agarose (Normand, V.; Lootens, D. L.; Amici, E.; Plucknett, K. P.; Aymard, P. *Biomacromolecules* **2000**, *1*, 730–738.) and (2) ultralow melting temperature agarose did not gel to a noticeable extent during the formation of the agarose film at 40 °C (Figure 2); instead, it remained dissolved in solution.

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indicating that the presence of (and interaction with) agarose did not result in immobilization of lipids in the liposome membranes.

After determining that agarose molecules associate with the membranes of giant liposomes during formation, we assessed the importance of the hybrid film of agarose and lipids for the formation of liposomes, in comparison to formation of liposomes from bare glass but in the presence of a solution that contained *soluble agarose* molecules. We explored two different approaches for using agarose in solution for the formation of giant liposomes. In the first approach, we exposed a lipid solution to agarose molecules before coating the lipids onto a glass slide to determine if this approach would lead to formation of giant liposomes. To carry out this experiment, we agitated a two phase system containing a 1% solution of ultralow melting agarose dissolved in deionized water and a solution of POPC lipids in chloroform and methanol. After vigorous shaking and allowing the immiscible fluids to separate, we extracted the chloroform/lipid solution and used it to coat plain glass slides with a film of lipids according to Figure 2B. We immersed the resulting lipid-coated slides in PBS and carried out formation of liposomes for 3 h. This method did not lead to significant formation of giant liposomes, indicating that, in order to form giant liposomes, agarose molecules had to be present either in the form of a film or in the aqueous solution during liposome formation. In order to distinguish between these remaining two possibilities, we carried out a second experiment that determined if association of agarose molecules dissolved in aqueous phase with lipid molecules during hydration would lead to formation of giant liposomes. We tested this possibility by coating plain glass slides with a solution of POPC lipids in chloroform and methanol. Subsequently, we immersed the coated slides in a PBS solution that contained dissolved ultralow melting temperature agarose at a concentration of 0.06% (w/w). This concentration was similar to the final concentration attainable if the slide had been coated with a film of agarose (as described in Figure 2) and if all of the agarose had dissolved; this concentration was also sufficiently low that ultralow melting agarose did not gel at room temperature. This second method also did not lead to significant formation of giant liposomes within 3 h. These experiments thus demonstrated that the formation of giant liposomes in PBS depended on the presence of agarose as a film prior to generating the hybrid film of agarose and lipids.

Based on the findings that (i) lipids infiltrate the films of agarose (Figure 4) during the coating procedure, (ii) agarose initially must be present as a film, and (iii) agarose molecules associate with lipid membranes after formation (Figure S5 of Supporting Information), we examined the possible benefits of agarose on each of the three stages of formation that are outlined in Figure 1. Due to the limited understanding of the molecular mechanisms of the formation of giant liposomes,<sup>23,33,38–40</sup> we cannot provide a definitive explanation of the role of films of agarose in the formation of giant liposomes. The work presented here does, however, examine several possible roles of agarose in promoting the formation of these liposomes, and it provides evidence in support of these roles.

With regard to stage 1, i.e., the proposed preorientation of lipids and formation of lamellae,<sup>1,10,11</sup> we hypothesized that the porous film of agarose with its large surface area may promote the formation of lamellae. Previous work indicated that the formation of lamellae may be influenced by interactions of the

lipids with the solid surface on which they are supported.<sup>1,63</sup> For instance, Angelova and Dimitrov reported that the lipid layers closest to a surface are structured differently than lipid layers located further away from the surface.<sup>1</sup> In the case of the hybrid film of agarose and lipids, Figure 4 shows that lipids infiltrate the porous film of agarose. It is reasonable to assume that the resulting large contact area between agarose molecules and lipid molecules affects the formation of lipid lamellae, especially when considering that the agarose film contained residual water.<sup>53,56,64–66</sup> Although the method described in Figure 2 involved partially drying the film of agarose for 1–3 h (until the film no longer appeared wet), we found that the film of agarose still contained ~15 wt% of water (see Supporting Information).

The residual water in the hybrid films of partially dried agarose and lipids may hence serve a similar function as the traditional prehydration step that is required by the protocols developed by Akashi et al.<sup>8</sup> and Yamashita et al.<sup>10</sup> Prehydration purportedly affects the orientation of lipids and the formation of lipid lamellae.<sup>8,10,11</sup> The residual water content in the partially dried film of agarose could thus provide this function during the formation of the lipid film from chloroform solutions and thereby eliminate the need for a separate prehydration step. To test this hypothesis, we varied the residual water content in films of agarose and compared the extent of formation of giant liposomes from the resulting hybrid films of agarose and lipids. First, we prepared particularly dry agarose films (water content <1 wt%, agarose content >99%) by keeping the agarose films overnight in an oven above 100 °C. Second, we prepared agarose films in the usual way (Figure 2) of drying at 40 °C for approximately 2 h (water content ~15 wt%, agarose content ~85%). And third, we prepared films of agarose in the usual manner (Figure 2) and then provided a separate prehydration step by exposing the hybrid film of agarose and lipids to water vapor in an enclosed chamber for 30 min. This prehydration procedure resulted in a water content of ~15–20 wt%. We found that the extent of formation of giant liposomes was reduced when the film of agarose was dried overnight at a temperature above the boiling point of water compared to the standard procedure as described in Figure 2. The liposomes that we did observe were typically smaller than 5  $\mu\text{m}$ . Interestingly, vapor-based prehydration of hybrid films of agarose and lipids that had been prepared in the usual manner (2 h drying at 40 °C) did not yield an increase in formation of giant liposomes. These results suggest that films of agarose, when dried partially at 40 °C for ~2 h, provided a similar effect as prehydration and that additional prehydration did not increase the benefit provided by the film of agarose alone.

With regard to stage 2, i.e., the growth of liposomes promoted by forces normal to the film of lipids,<sup>3,42</sup> it is important to consider the hybrid nature of the films of partially dried agarose and lipids (Figure 2 and Figure 4). We hypothesize that, as soon as water enters this hybrid film, agarose molecules within the

(63) LeBerre, M.; Yamada, A.; Reck, L.; Chen, Y.; Baigl, D. *Langmuir* **2008**, *24*, 2643–2649.

(64) Itagaki, H.; Fukiishi, H.; Imai, T.; Watase, M. *J. Polym. Sci., Part B: Polym. Phys.* **2005**, *43*, 680–688.

(65) Itagaki et al. provide additional evidence of water molecules co-crystallizing with agarose by use of a fluorescent probe (Itagaki, H.; Fukiishi, H.; Imai, T.; Watase, M. *J. Polym. Sci., Part B: Polym. Phys.* **2005**, *43*, 680–688).

(66) Arndt and Stevens discuss the presence of tightly bound water molecules within dried agarose gels as determined by vacuum UV CD (Arndt, E. R.; Stevens, E. S. *Biopolymers* **1994**, 341527–1534).



hybrid film of agarose and lipids dissolve and hence generate hyperosmotic conditions during the initial stages of liposome formation when only small amounts of water are present between the lipid lamellae. If this hypothesis is correct, then the resulting hyperosmotic conditions would lead to an influx of water<sup>11,67</sup> and thus generate forces normal to the lipid lamellae in the hybrid film, and furthermore a film of a cross-linked hydrogel would provide a reduced benefit compared to a hydrogel that can dissolve into many individual molecules. We found that a chemically cross-linked polyacrylamide gel facilitated the formation of giant liposomes in solutions of physiologic ionic strength, but typically to a smaller extent than formation from ultralow melting temperature agarose (see Supporting Information, Figure S7). In addition, the formation of giant liposomes worked best when the agarose films were formed with a type of agarose that dissolved in water and, hence, increased the number of agarose molecules in solution; this increased number of molecules affects colligative properties, such as osmotic pressure. For instance, agarose with ultralow and, to a smaller extent, with low melting temperature generated the highest yield of giant liposomes in solutions of physiologic ionic strength, whereas dried films of standard or high melting temperature agarose did not provide a substantial benefit compared to formation from films of lipids supported on bare glass or ITO (see Supporting Information, Figure S7). We observed that ultralow and low melting agarose dissolved partially during one hour of liposome formation, while films from standard and high melting temperature agarose did not dissolve noticeably. Moreover, swelling of films from standard and high melting temperature agarose proceeded more evenly and slowly (over the course of 1 h) than swelling of films of ultralow and low melting agarose types, which occurred within the first few seconds of formation.

One additional effect that could promote stage 2 of liposome formation is that the presence of partially hydrophilic agarose molecules in this heterogeneous film of agarose and lipids might promote the influx of water into the lipid film.<sup>11,67</sup> Such facilitated hydration may further promote the separation of lipid bilayers in the nascent liposomes and generate forces normal to the lipid bilayers. To test this hypothesis, we immersed glass slides containing a film of agarose in PBS for 30 s. Films of ultralow melting and of low melting agarose appeared to hydrate completely during this short immersion in PBS; these hydrated, swollen, and partially dissolved films could be displaced from the surface of the glass by a stream of pressurized air. In contrast, films of standard and high melting temperature agarose did not swell noticeably during the 30 s immersion in PBS<sup>68</sup> and remained as thin films that adhered firmly to the surface of the glass; these films did not dissolve noticeably within 1 h.

Phase contrast microscopy observations during the early stages of liposome formation provided additional evidence for swelling of films of agarose after immersing the slides in aqueous solution.<sup>69</sup> Specifically, this swelling increased the thickness of the film and hence moved the nascent layer of liposomes out of the focal plane of the microscope away from the surface of the glass. Given that the entire film swelled

observably, agarose molecules must have undergone translational motion at a microscopic level. This conclusion agrees with findings by Fialkowski et al., who related the influx of water to increased elastic energy in hydrogels.<sup>67</sup> Since the films were composed of both agarose and lipids, it follows that forces acting on the agarose film also acted directly or indirectly on lipids and on lipid lamellae in the film. In this sense, the swelling hydrogel film may provide benefits similar to the forces provided by alternating electric fields in electroformation.<sup>42</sup>

In order to investigate if applying alternating electric fields would provide an additional benefit, we formed giant liposomes in deionized water from hybrid films of agarose and lipids with and without an applied AC electric field as well as from ITO plates without agarose in the presence (electroformation) and absence (gentle hydration) of an electric field. We found that, in deionized water, the yield of giant liposomes from hybrid films of agarose and lipids was not affected by the presence of an applied AC electric field; it was similar to the yield from standard electroformation. In contrast, the yield of giant liposomes by gentle hydration from bare glass surfaces without electric fields was significantly lower (see Supporting Information, Figure S8) compared to formation from hybrid films of agarose and lipids. Importantly, standard electroformation did not generate giant liposomes in PBS whereas giant liposomes formed readily in PBS from hybrid films of agarose and lipids, and they did so with or without an electric field.

With regard to stage 3, i.e., the fusion of adjacent liposomes, time-lapse series of phase contrast micrographs revealed numerous fusion events of giant liposomes in PBS solutions, in particular during the first minutes of formation (Figure 6). We did not detect such fusion events in control experiments with lipid films on bare glass, presumably due to the low density of liposomes that formed on bare glass in PBS. Films of agarose hence strongly promoted fusion of liposomes<sup>70</sup> probably due to crowding and the associated mechanical stresses<sup>46</sup> of the large number of liposomes that grew outward from the porous agarose film into the solution above the hybrid films and possibly due to mechanical stresses generated by the swelling hydrogel film.<sup>41</sup>

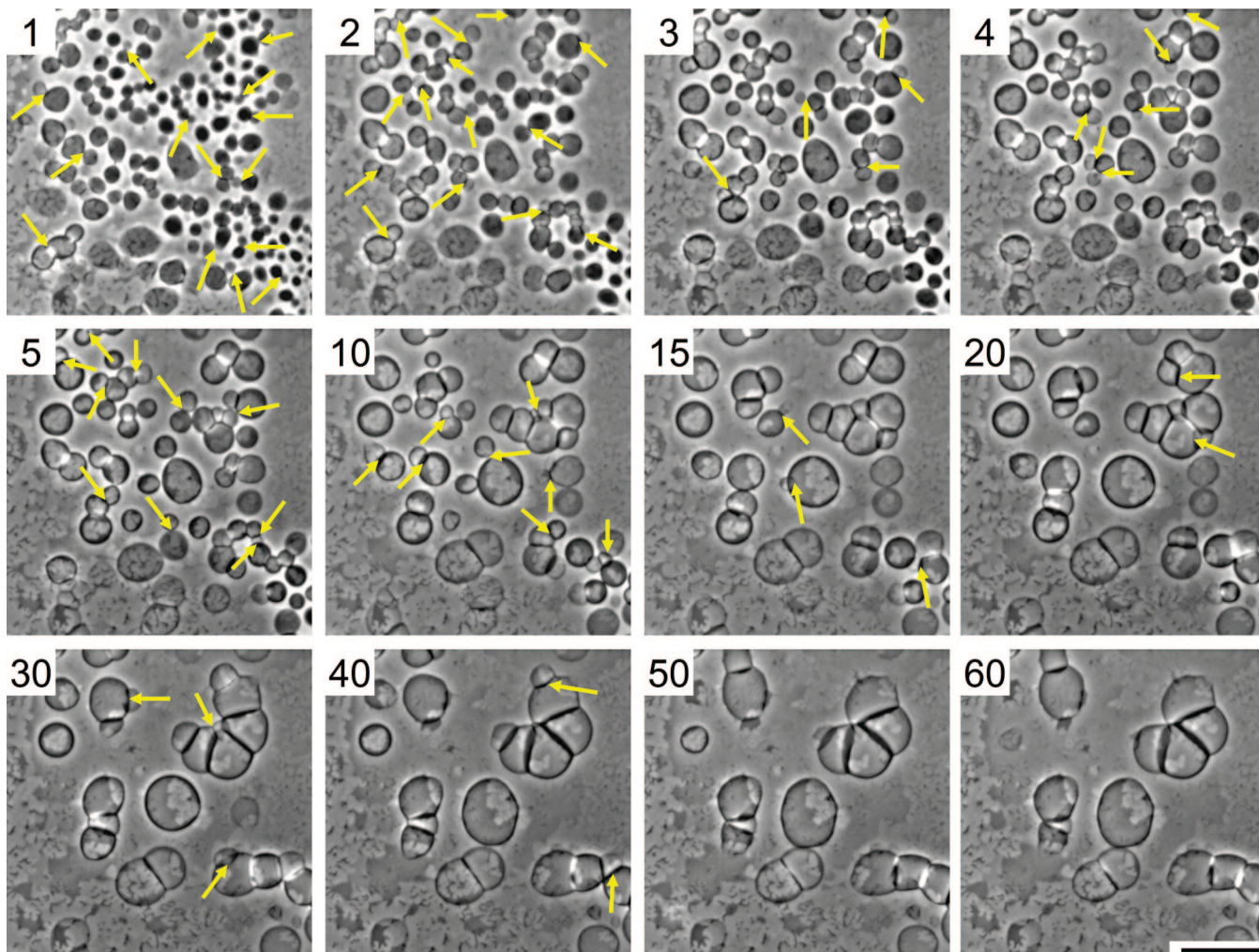
(67) Fialkowski, M.; Campbell, C. J.; Bensemann, I. T.; Grzybowski, B. A. *Langmuir* **2004**, *20*, 3513–3516.

(68) In fact, even after 2 min of hydration, high melting agarose appeared to remain as a dry film.

(69) We followed the swelling by adjusting the focal plane of the microscope as the hybrid film of agarose and lipids moved away from the surface of the glass slide.

(70) One may ask if liposomes grown from hybrid films of agarose and lipid formulations that contained negatively charged lipids or PEGylated lipids (Figure 3C, E, G, M, O, Q, S) also fused during the formation process. In this context, it is instructive that in other methods of formation, such as electroformation or freeze-and-thaw, fusion of adjacent liposome membranes is also a key characteristic in formation of giant liposomes (Menger, F. M.; Angelova, M. I. *Acc. Chem. Res.* **1998**, *31*, 789–797; Castile, J. D.; Taylor, K. M. G. *Int. J. Pharm.* **1999**, *188*, 87–95; Oku, N.; Macdonald, R. C. *Biochemistry* **1983**, *22*, 855–863 and these methods also yield giant liposomes from lipid compositions that include PEGylated lipids. (Constantinescu, I.; Levin, E.; Gyongyossy-Issa, M. *Artif. Cells Blood Substit. Immobil. Biotechnol.* **2003**, *31*, 395–424) or anionic lipids. (Claessens, M.; Leermakers, F. A. M.; Hoekstra, F. A.; Stuart, M. A. C. *J. Phys. Chem. B* **2007**, *111*, 7127–7132). Therefore, the repulsive forces generated by these specialized lipid formulations, which are often used to promote initial bilayer orientation and separation, can be overcome by appropriate mechanical stresses. (Angelova, M. I.; Dimitrov, D. S. *Prog. Colloid Polym. Sci.* **1988**, 7659–67; Cevc, G.; Richardsen, H. *Adv. Drug Deliv. Rev.* **1999**, *38*, 207–232) such as those generated by an electric field, osmotic pressure during freeze-and-thaw cycles, or as proposed here, by a swelling hydrogel film.

(71) A definite analysis of lamellarity is typically obtained by micropipette aspiration to measure the bending modulus (Needham, D.; McIntosh, T. J.; Lasic, D. D. *Biochim. Biophys. Acta* **1992**, *1108*, 40–48; Akashi, K.; Miyata, H.; Itoh, H.; Kinoshita, K. *Biophys. J.* **1996**, *71*, 3242–3250; Yamashita, Y.; Oka, M.; Tanaka, T.; Yamazaki, M. *Biochim. Biophys. Acta* **2002**, *1561*, 129–134; Angelova, M.; Soleau, S.; Meleard, P.; Faucon, J. F.; Bothorel, P. *Prog. Colloid Polym. Sci.* **1992**, *89*, 127–131; Bermudez, H.; Hammer, D. A.; Discher, D. E. *Langmuir* **2004**, *20*, 540–543).



**Figure 6.** Time-lapse series of phase contrast micrographs during the formation of giant liposomes in PBS from a hybrid film of ultralow melting temperature agarose and pure POPC lipids. Image capture began within seconds after adding PBS to the formation chamber and the micrographs depict the same location on the glass slide throughout the entire time series. Yellow arrows indicate fusion events that occurred before the next time point of image capture. Numbers in the upper left corner of each frame indicate elapsed time in minutes from the start of the time series. Scale bar = 100  $\mu\text{m}$ .

Figure 6 also illustrates that films of agarose promoted rapid formation of giant liposomes in PBS: typically, giant liposomes formed within 5 min upon addition of aqueous solutions to hybrid films of agarose and lipids.

One important aspect of the method presented here is the lamellarity of the resulting giant liposomes in solutions of physiologic ionic strength.<sup>71–73</sup> In order to address this question, we analyzed the fluorescence intensity of the membranes using a confocal microscope.<sup>8,10</sup> We formed liposomes composed of POPC doped with 1% 1,2-dipalmitoyl-*sn*-glycero-3-phospho-

ethanolamine-*N*-(lissamine rhodamine B sulfonyl)(ammonium salt) (referred to as DPPE-rhodamine) and liposomes composed of POPC with 5% PEG-PE and 1% DPPE-rhodamine in PBS from films of ultralow melting temperature agarose. We measured the fluorescence intensity at multiple locations on the membranes of free-floating giant liposomes procured from these preparations and determined the size and average intensity of each liposome (Figure 7).

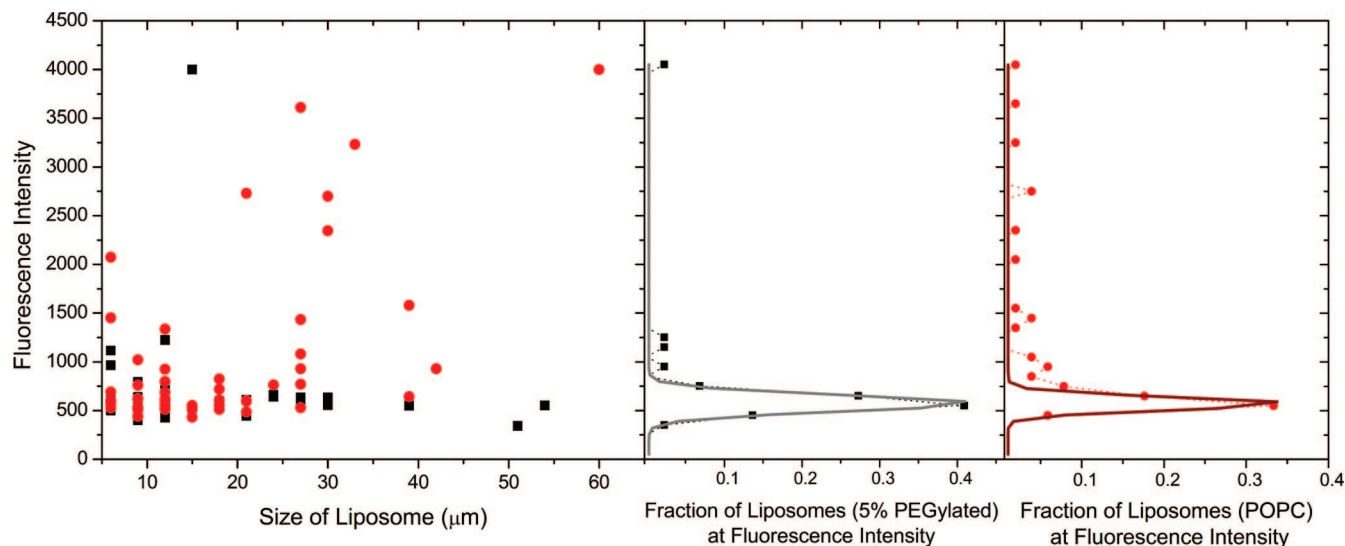
Akashi et al. showed previously that the fluorescence intensity of unilamellar liposomes varied with the size of the liposomes. In order to account for this effect, we constructed histograms of fluorescence intensity and carried out a best curve fit with a Gaussian function. We conducted this analysis first on liposomes that contained 5% PEGylated lipids. Liposomes with such a high content in PEGylated lipids are predominantly unilamellar, even in the presence of ionic solutions.<sup>10,44</sup>

Figure 7 shows that almost all giant liposomes with 5% PEGylated lipids had fluorescence intensities between 300 and 750 arbitrary units and that a Gaussian curve with a single maximum at  $\sim 570$  arbitrary units fit the data very well. Figure 7 also shows that the same analysis on POPC liposomes (without PEGylated lipids) that were formed from agarose films in PBS solution also yielded a peak with a maximum intensity at  $\sim 570$

(72) Measuring the bending modulus by micropipet aspiration is difficult to perform and requires specialized equipment and expertise (Hotani, H.; Nomura, F.; Suzuki, Y. *Curr. Opin. Colloid Interface Sci.* **1999**, *4*, 358–368. Akashi, K.; Miyata, H.; Itoh, H.; Kinoshita, K. *Biophys. J.* **1996**, *71*, 3242–3250. Yamashita, Y.; Oka, M.; Tanaka, T.; Yamazaki, M. *Biochim. Biophys. Acta* **2002**, *1561*, 129–134. Bermudez, H.; Hammer, D. A.; Discher, D. E. *Langmuir* **2004**, *20*, 540–543. Farge, E.; Deveaux, P. F. *Biophys. J.* **1992**, *61*, 347–57).

(73) The bending modulus can be affected by ions in the solution (Dimova, R.; Aranda, S.; Bezlyepkina, N.; Nikolov, V.; Riske, K. A.; Lipowsky, R. *J. Phys.: Condens. Matter* **2006**, *18*, S1151–S1176) by macromolecules associated with the membrane, such as PEG. (Yamashita, Y.; Oka, M.; Tanaka, T.; Yamazaki, M. *Biochim. Biophys. Acta* **2002**, *1561*, 129–134. Bermudez, H.; Hammer, D. A.; Discher, D. E. *Langmuir* **2004**, *20*, 540–543), or possibly by agarose.





**Figure 7.** Average fluorescence intensity of giant liposomes as a function of liposome diameter and corresponding Gaussian fits to the distribution in fluorescence intensity. (Left) Average fluorescence intensities of free-floating liposomes formed for three hours in PBS from hybrid films of ultralow melting temperature agarose and lipids. Black squares represent giant liposomes composed of POPC with 5 mol% PEGylated lipids and 1% DPPE-rhodamine; red circles represent giant liposomes composed of POPC with 1% DPPE-rhodamine. Each symbol in the left panel corresponds to one liposome (total count = 95; 51 were composed of POPC and 44 contained PEGylated lipids). (Center) Distribution of the average fluorescence intensities of giant liposomes composed of POPC with 5 mol% PEGylated lipids and 1% DPPE-rhodamine (indicated by the black squares connected with a dashed black line) and the fit of a Gaussian function to the main peak of intensities (solid gray line). (Right) Distribution of the average fluorescence intensities of giant liposomes composed of POPC with 1% DPPE-rhodamine (indicated by the red circles connected with a dashed red line) and the fit of a Gaussian function to the main peak of intensities (solid red line). Note that the camera saturated at fluorescence intensities above 4000.

arbitrary units. This result suggests that a significant fraction of free-floating giant liposomes was unilamellar when these liposomes were formed from agarose in PBS, even in the absence of PEGylated lipids. Figure 7 also shows, however, two additional small peaks of fluorescence intensity that appeared in the histograms of POPC liposomes at  $\sim 1000$  arbitrary units and at  $\sim 1500$  arbitrary units (dashed red line). These intensities suggest the presence of two and three bilayers in the liposomes, respectively. Moreover, this preparation also contained liposomes with fluorescence intensities that indicate a larger number of bilayers than three. Formation of giant liposomes from hybrid films of agarose and lipids hence appeared to generate both unilamellar and multilamellar liposomes in PBS solutions.

One possible application of giant liposomes in physiologic buffer is to carry out reactions inside these liposomes. To this end, it would be useful to encapsulate molecules inside the liposomes. We tested this possibility by forming giant liposomes from hybrid films of agarose and lipids in a flow chamber<sup>22</sup> in a solution that contained 0.1 mM Tris (pH 7.4) with 0.5  $\mu$ M dextran (MW 70 000) labeled covalently with fluorescein isothiocyanate (FITC). After 3 h of formation, we replaced the solution in the chamber with a 0.1 mM Tris solution (pH 7.4) that did not contain FITC-conjugated dextran. We found that giant liposomes, when formed from hybrid films of agarose and lipids, did encapsulate FITC-conjugated dextran but at a lower concentration inside the liposomes than was present in the surrounding solution (see Supporting Information, Figure S9). In addition, a fraction of the encapsulated dextran remained in giant liposomes after the solution was exchanged. These results demonstrated that this method of forming giant liposomes from films of agarose may be useful for encapsulating large water-

soluble molecules within giant liposomes, thus opening up the possibility to use them as microreactors.

## Conclusion

Gentle hydration of hybrid films of partially dried agarose and lipids provides a straightforward, rapid, and reproducible procedure to generate giant liposomes in high yield in solutions of physiologic ionic strength. This method does not require any specialized equipment and makes it possible to generate giant liposomes in PBS from a variety of lipids, including lipid compositions that typically pose problems for formation of giant liposomes, such as pure phosphatidylserine, pure phosphatidylglycerol, or asolectin. During the formation process, agarose molecules associate with the lipid membranes of the resulting liposomes while the lipids in the liposomes maintain fluidity. This interaction of macromolecular carbohydrate molecules with liposome membranes may have a similar beneficial effect as PEGylated lipids for the formation of giant liposomes in solutions with physiologic ionic strength. Analysis of free-floating giant liposomes prepared from hybrid films of partially dried agarose and lipids revealed that this method appeared to generate both unilamellar and multilamellar giant liposomes in PBS, even in the absence of PEGylated or charged lipids. The short time of formation, reliability, and high yield of the simple method presented here is appealing for generating giant liposomes in physiologic solutions and provides the opportunity to extend studies with giant liposomes to physiologically relevant conditions. Moreover, the remarkably strong benefit of hybrid films of agarose and lipids with regard to the yield of giant liposomes in ionic solutions supports several proposed mechanisms of formation of these important models of cell membranes<sup>1,3,10,41,42</sup> and may thus contribute to the understanding of the formation of giant liposomes.

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**Supporting Information Available:** Experimental details including materials and methods, yield of free-floating giant liposomes that were formed from films of agarose, formation of giant liposomes in three types of aqueous solutions, surface

topography of films of different types of agarose as characterized by SEM and AFM, fluorescence intensities of giant liposomes formed from a hybrid film of fluorescently labeled agarose and lipids and from a hybrid film of nonlabeled agarose and lipids, determination of membrane fluidity by fluorescence recovery after photobleaching (FRAP), residual water content of partially dried agarose films, formation of giant liposomes from a hybrid film of cross-linked hydrogel and lipids as well as from hybrid films of other types of agarose and lipids, effect of an electric AC field on the formation of giant liposomes from hybrid films of agarose and lipids, and encapsulation of water-soluble macromolecules into giant liposomes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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