

# Cationic Lipid Absorption on Titanium: A Counterion-Mediated Bilayer-to-Lipid-Tubule-Network Transition

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By carefully tuning the screening effects of monovalent salt in solution, it is possible to control the formation of different lipid morphologies on a titanium dioxide substrate. A transition from an adsorbed vesicle layer to fluid lipid bilayers is observed as a function of monovalent salt concentration in solution, and, above a threshold salt concentration, a network of lipid tubules with a fascinating distorted-hexagonal structure is formed in two dimensions on the substrate. We present fluorescence microscopy data for these different morphologies, confirming the connectivity and fluidity of the bilayers and tubules.

Lipids are amphiphilic molecules and have been observed to form a variety of different structures in solution. A rich lyotropic phase sequence can be observed in lipid systems, including micellar, lamellar, and hexagonal phases. Of particular recent interest are lipid tubules.<sup>1</sup> These cylindrical structures, often composed of chiral bilayers wrapped into a tube structure (similar to a paper drinking straw)<sup>2,3</sup> have been observed to form in a variety of materials. The tubules are hollow and may be multi- or unilamellar.

Some research has been done to try to control the formation of lipid tubules;<sup>4,5</sup> various strategies include growing individual oriented tubules<sup>6</sup> or connecting vesicles with tiny lipid tubules.<sup>7</sup> Polymerizable lipids can be used, whereby the tubules are essentially “fixed” after formation and can be dried. This fixing has allowed the possibility of more detailed scanning electron microscope characterization and their use in applications such as conduits between vesicle “bioreactors”.<sup>7</sup>

The surface properties of titanium constitute an important topic for research, as this metal is one of the most biocompatible materials in use today. Titanium is commonly used for the construction of medical implants and is often employed as a coating material for devices implanted into the body due to its very low interaction with the cellular environment in comparison to other potential implant materials. The native titanium dioxide (TiO<sub>2</sub>) layer on bulk titanium is responsible for its biocompatibility and exhibits a negative surface charge. Despite its similarity to silicon dioxide (SiO<sub>2</sub>), it has not been easy to form lipid bilayers on the TiO<sub>2</sub> surface. The first report of such a structure was reported recently with anionic lipid mixtures in the presence of calcium ions.<sup>8</sup> The development of such strategies will open the door for numerous applications in medical implants, including new drug delivery systems and built-in biological sensors.

In this paper we present the first work in which a bilayer has been formed on TiO<sub>2</sub> using cationic lipid mixtures, the fluidity of which is confirmed by fluorescence recovery after photobleaching (FRAP) measurements. In addition, by controlling the ionic concentration of the lipid solution, a striking phenomenon has been observed, in which a two-dimensional (2D) network of lipid tubules is trapped on the TiO<sub>2</sub> surface. This tubule network exhibits surprising hexagonal loops and continuous fluid characteristics.

## Experimental Methods

Lipid mixtures of cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (both from Avanti Polar Lipids) were prepared in chloroform at varying molar ratios. These mixtures were then dried under nitrogen and overnight vacuum, then rehydrated at various salt concentrations with Millipore water or with phosphate buffered saline (PBS)<sup>9</sup> to a 0.6 mM lipid concentration. The lipids were verified to be well mixed by solution X-ray diffraction. DOTAP is a cationic lipid with an overall positive charge on the headgroup, thus, by varying the molar ratio of DOTAP to DOPC, the charge density of the lipid bilayer is varied. For fluorescence measurements, a small amount (~1% of the total lipid weight) of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine labeled with Texas Red (Molecular Probes, Inc.) was included in the mixtures. Unilamellar vesicles with an average diameter of ~100 nm were prepared by tip sonication. Vesicle size was verified by dynamic light scattering. All measurements described in this paper were carried out at room temperature, well above the lipid melting temperature ( $T_m$ ) for both DOPC and DOTAP.

## Results and Discussion

Highly polished Ti substrates were used for this work, with a surface roughness of ~3 nm, as measured by contact-mode atomic force microscopy. While relatively rough compared with atomically smooth substrates such as mica, this represents an extremely smooth polished metallic surface.

Lipid deposition on the titanium substrate was carried out via the vesicle fusion method: small unilamellar vesicles are prepared and deposited on the substrate surface at room temperature, then rinsed after a certain incubation time. The vesicles may fuse onto the surface to form a bilayer. In the work presented here, a 1 mL droplet of lipid solution was deposited on the 2 cm × 2 cm Ti surface and rinsed after 60 min. To promote absorption, the

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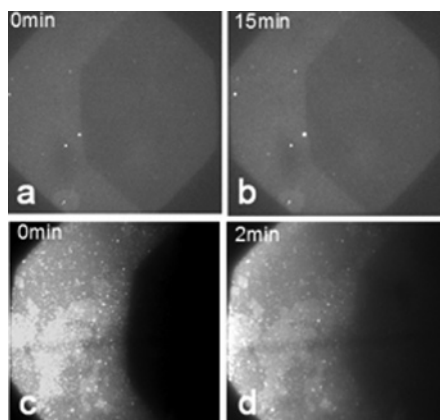
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(9) 137 mM NaCl, 2.7 mM KCl, 11.9 mM phosphates.



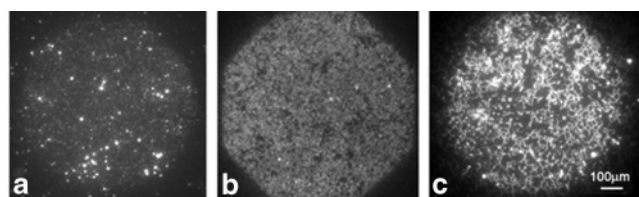
**Figure 1.** FRAP data comparing the absorbed vesicle layers at 0 mM NaCl (a,b) (no fluorescence recovery observed), with the absorbed bilayer at 100 mM NaCl (fluorescence recovery) (c,d).

hydrophilicity of the native TiO<sub>2</sub> layer was increased by using an O<sub>2</sub> plasma treatment for 3 min.

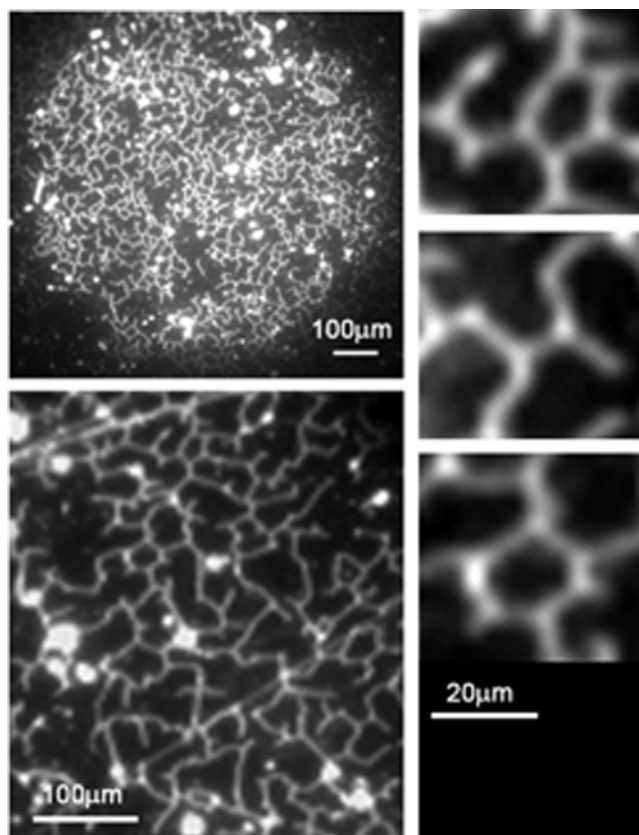
The rinsing and sample preparation technique was extremely important, as it was found that the tubule networks were fairly loosely bound to the titanium dioxide surface. Rinsing was carried out gently in a large water bath and samples were carefully sealed, taking care not to let the surface dry out. We observed that the capillary forces, which often act on solution samples in the process of microscope slide sealing, induce a strong fluid flow. This flow may be enough to strip the absorbed lipid from the Ti surface. This is understandable, as a cylindrical tubule will have a small point of contact with a flat surface compared with that of a flat bilayer, and drying out would clearly have a detrimental effect on the absorbed structures.

Three different lipid morphologies have been observed on the TiO<sub>2</sub> surface, controlled by the concentration of monovalent salt in the solution with which the lipids were rehydrated and the vesicles were formed. In previous work, researchers attempted to form a bilayer on TiO<sub>2</sub> substrates such as these but without success; instead, an absorbed vesicle layer was formed.<sup>10–12</sup> Small vesicles attach to the surface, giving the visual appearance of a bilayer; however, FRAP measurements reveal that the absorbed lipid is not continuous and no fluorescence recovery is observed. This absorbed vesicle layer has been observed in 1:1 DOTAP/DOPC lipid mixtures at low salt concentrations and in pure water, in good agreement with the previous observations of other authors. Figure 1 (panels a and b) shows FRAP data for a sample prepared in Millipore water (no NaCl). An absorbed lipid layer on the TiO<sub>2</sub> was imaged after an area was fluorescence bleached at  $t = 0$  min and then at  $t = 15$  min. After 15 min, no fluorescence recovery was observed in the bleached area. This result indicates that the lipid on the surface is not a continuous fluid layer at 0 mM NaCl.

If the NaCl concentration is increased to 100 mM, a different morphology is observed on the surface, which appears to be a patchy bilayer. The continuous nature of this bilayer was confirmed by FRAP imaging, where we observed fluorescence recovery (Figure 1). This result indicates that the absorbed layer has fluid-like properties and is distinct from the vesicle layer seen at low salt concentrations. The patchy bilayers observed here are reminiscent of structures observed on mica surfaces recently,<sup>13,14</sup> although, clearly, substrate inhomogeneities will



**Figure 2.** Fluorescence microscopy images of different lipid morphologies on TiO<sub>2</sub>: (a) absorbed vesicle layer at 50 mM NaCl, (b) rough fluid bilayer at 100 mM NaCl, and (c) tubule network at 150 mM NaCl.



**Figure 3.** Cationic lipid tubules on the same TiO<sub>2</sub> surface at different magnifications prepared in PBS buffer.

play an important role in the bilayer coverage due to phenomena such as pinning and contact-angle variations.<sup>15</sup>

The most exciting observation occurs when samples are prepared at salt concentrations of 150–200 mM NaCl. A network of lipid tubules forms in two dimensions on the surface of the titanium. In addition, the tubule network appears to have a degree of hexagonal ordering with a preferred angle of branching between the tubules. Figure 2 shows a comparison between the three different lipid morphologies observed in this experiment as a function of monovalent salt concentration. Similar behavior is also observed upon rehydration with PBS solution,<sup>9</sup> the composition of which is mostly monovalent salt with some other components. FRAP experiments on this network demonstrate the fluidity of the tubules on the surface. Figure 3 shows, in more detail, the tubule network, including examples of the remarkable hexagonal structures.

By modulating the ionic environment of the vesicles, it is possible to control not only absorption to the surface, but also

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lipid bilayer morphology. It is clear that the concentration of monovalent ions in solution is a key to the formation of different structures. In addition, the charge density of the lipid bilayer plays an important role. The interaction of ions in solution with charged particles and surfaces is a subject of general interest, and, in this experiment, several different charge interactions come into play: mutual interactions between charged spheres (i.e., the vesicles), interactions between these vesicles and the charged substrate surface, and interactions of both the vesicles and the surface with the counterions in the solution. In addition, the phenomenon of vesicle/tubule fusion must also be taken into account. The effect of ionic species in solution on cationic tubule formation has not been investigated in detail and warrants a more complete study.

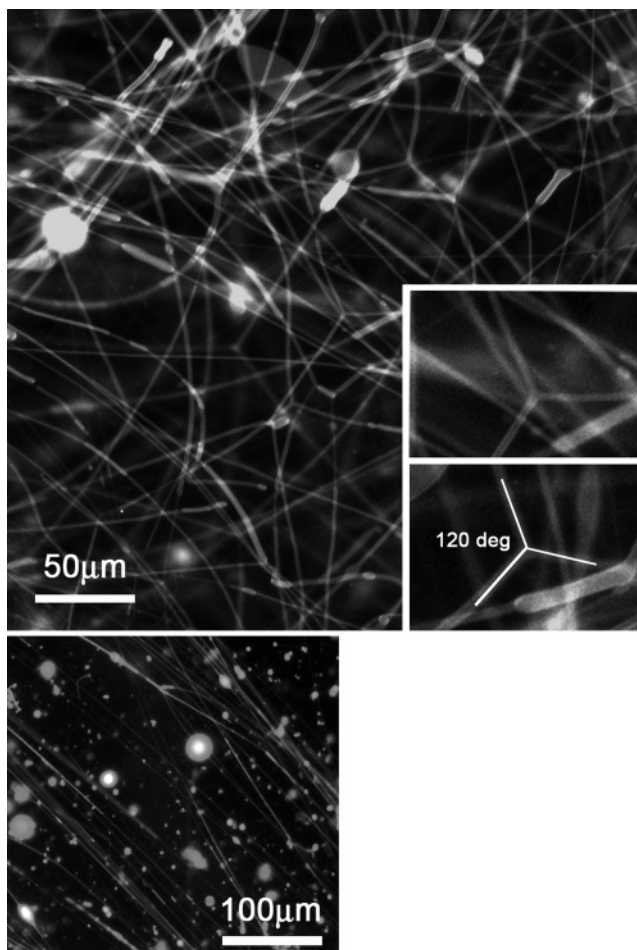
The TiO<sub>2</sub> surface is known to be negatively charged, and it was initially assumed that cationic molecules would readily be attracted to the surface. The interactions in this system, however, appear to be much more complicated. It is not enough to just prepare cationic vesicles to induce bilayer formation on titanium: in pure water, the charged vesicles do not readily fuse with each other, due to their repulsive interactions, and instead form an adsorbed vesicle layer as described by previous authors.<sup>10–12</sup> By using vesicles formed in monovalent salt solution, one may utilize screening effects to modulate this surface absorption. At low salt concentrations of 50–100 mM NaCl, vesicle fusion is promoted as the like-charge repulsion between vesicles is screened, and they can, on average, move closer to each other. Thus, a bilayer is able to form on the TiO<sub>2</sub> surface.

Increasing the ionic concentration still further, tubule attachment to the surface occurs. The formation of lipid tubules on the substrate surface is very sensitive to the concentration of ions in solution. The positively charged tubules are electrostatically attracted to the TiO<sub>2</sub> surface and remain loosely bound in the presence of monovalent salt at 150–200 mM. If NaCl concentration is increased further to >300 mM, tubules are no longer observed, and once again an adsorbed vesicle layer will form. Experimentation with different charge densities is still in the preliminary stages, although it has been observed that tubule formation on the substrate does not occur at a ratio of 1:3 DOTAP/DOPC.

The tubules observed on the TiO<sub>2</sub> surface are short, unlike those typically observed in bulk, and display a fascinating hexagonal arrangement, as shown in Figure 3. Their arrangement is fairly disordered; however, it is clear that they exhibit a preferred angle of branching, close to 120°.

Branching in lipid tubules has been observed recently<sup>16,17</sup> by deliberately manipulating lipid tubules extruded from unilamellar vesicles and causing them to fuse with each other. In this way, specific networks can be formed, linking vesicles together by narrow conduits. We have observed lipid tubule fusion in bulk solutions of DOPC tubules (Figure 4a) by controlled flow. In the image shown in Figure 4a, many junctions are observed with a preferred branching angle of ~120°. It is clear from these bulk experiments that this optimal branching angle is a universal property for the three-way lipid junction. When two tubules fuse, they must minimize their curvature at the junction. This is achieved by taking an angle of 120° between branches.

DOPC/DOTAP tubules are not typically stable in solution, and we have observed that, after their generation by hydrodynamic flow (Figure 4b), they gradually deform to become spherical vesicles.



**Figure 4.** Fluorescence microscopy image of (a) branching lipid tubules formed in solution from DOPC and (b) lipid tubules in 50% DOPC, 50% DOTAP.

It is interesting to note that, if the same lipid mixtures are prepared on a glass substrate, the tubule phenomenon is not observed. A negatively charged glass surface readily attracts cationic lipids to form a bilayer, and it is clear that the solution tubules are attracted to a glass surface, where they rapidly fuse and become incorporated into a bilayer. The TiO<sub>2</sub> substrate clearly does not induce tubule formation; however, it appears to stabilize the tubules on its surface in a way that glass is unable to. Tubules are trapped electrostatically but do not fully fuse, similar to the adsorbed vesicle layer. Some junctions are able to form, however, by tubule fusion, creating the hexagonal motif. In a delicate balance of charge interactions, this result is only possible by fine-tuning the vesicle fusion process using the screening effects of charges in solution.

There are several physical factors that come into play in defining the critical salt concentrations for vesicle fusion and bilayer/tubule formation. As discussed above, the charge density of the vesicle surface appears to be the dominant factor; however additional parameters may be controlled to further investigate this phenomenon. Temperature may affect the process significantly, as thermal fluctuations increase the likelihood of vesicle interaction and fusion taking place. Indeed, this effect has been observed on SiO<sub>2</sub> surfaces,<sup>10</sup> where higher temperatures increase the rate of vesicle fusion and bilayer formation. Additionally, vesicle size and flexibility may affect the threshold point. Larger vesicles are able to spread onto a surface if they are strongly attracted, forming high curvature regions at the edge of the contact point and increasing the likelihood of rupture. Flexibility of the

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vesicle wall may also be an important parameter for the formation of tubules on the surface. Not all lipids will readily form stable tubules, due to the high bilayer curvature required for tubule formation.

### Conclusion

In summary, we present the controlled formation of lipid structures on  $\text{TiO}_2$ . Using cationic lipid mixtures of DOTAP and DOPC at different monovalent salt concentrations, we are able to modulate the lipid structures formed, from an absorbed vesicle layer, to fluid bilayers, to a lipid tubule network with distorted hexagonal ordering.

By adding a small amount of counterions to the solution, we find that vesicle fusion may be triggered on the surface, and a fluid bilayer will form, albeit rough in comparison to bilayers typically observed on glass and mica. This roughness can most likely be attributed to the roughness of the substrate. At higher concentrations of counterions, we induce a new 2D networked tubule phase on the surface of the titanium. Tubules are observed in solution for this cationic mixture, induced by hydrodynamic

flow; however, they are not stable and are accompanied by many different vesicle structures. By electrostatically trapping the tubules on the Ti surface, we are able to stabilize a fascinating hexagonal structure.

Control of lipid tubule architectures has been of interest recently, as a method of constructing microscale reactors for chemical and biochemical processes. If such structures can be applied to a surface in a controlled fashion, essentially trapping difficult to handle three-dimensional structures in two dimensions, this will greatly increase the usability of such a system. In this paper we have demonstrated that this is certainly possible by the controlled use of electrostatics.

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