

Bionanotubule Formation from Surface-Attached Liposomes Using Electric Fields

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Received September 3, 2008. Revised Manuscript Received October 9, 2008

Spontaneous formation of long-range (millimeters) membrane-bound nanotubules from surface-immobilized liposomes is possible by application of modest electric fields (2–20 V/cm), providing a novel fabrication strategy for these hollow cylindrical structures. Stable tubes generally aligned with the applied electric field were created from liposomes prepared with phosphatidylcholine (PC), phosphatidic acid (PA), phosphoethanolamine (PE), and cholesterol. The minimum voltage which causes nanotubular formation (the onset voltage) and the average number of tubules per liposome of varying composition was examined with fluorescent microscopy using labeled phospholipids. Generally, the onset voltages ranged between 4 and 15 V/cm and depended on the mother vesicle composition. The results of this study suggest that increasing the charged lipid content can decrease the onset voltage. Conversely, a cholesterol content of more than 30% (by mass) was found to hinder extension of lipid tubules. Basic calculations that assume lipid migration and domain formation on the mother liposome as a nucleating site for tubule extension are assessed and suggest this is a reasonable model to describe the mechanism of tubular growth from immobilized liposomes.

Introduction

Synthetic lipid nanotubules were first created more than a decade ago,^{1,2} while the natural occurrence and function of these structures are still being discovered.^{3–8} Naturally occurring bionanotubules span lengths in the hundreds of micrometers, have diameters of 20–50 nm, and are capable of shuttling material between cells. They demonstrate a functional role in immunity in addition to being observed in other systems.^{3,4} The synthetic tubules are attractive for many fundamental and technological applications, including development of useful micro- and nanoscale networks.⁹

Recently, advances in the rational control of tubular structures have been achieved by adjusting chemical composition and conditions of growth.^{10–20} Many techniques are used to create

lipid nanotubules, including microfluidic devices,^{7,8} micromanipulation protocols such pipet aspiration and electroinjection techniques,^{21,22} high-pressure shearing,²³ and self-assembly in aqueous dispersions.^{1,11,24} They produce tubules from many different phospholipids including phosphatidylcholines,^{7,21,22} glycolipids,^{24,25} and sphingolipids.²³ Understanding and controlling the alignment of lipid tubules on substrates using these techniques as well as other procedures (e.g., magnetic alignment of lipid nanotubules in solution²⁶) have also been investigated. The physics of lipid bilayer-bound tubules, whether formed via biological actions or mechanical motion, have been thoroughly examined, and they are considered stable structures constituting a local minima within the available configurations of lipid bilayers.^{6,27,28} Long-range complex intertwined structures have been observed including nanotubules forming from a free colloid in a higher magnitude electric field (300–500 V/cm).²⁹ Other studies applying an electric field are generally limited to cellular systems and application of alternating voltage or large magnitude field strengths to induce porosity or rupture the membrane.^{30,31}

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The work presented here is the result of application of a modest (1–20 V/cm) electric field on micrometer-size liposomes electrostatically attached to a glass substrate. Tubular formation at different lipid ratios is examined as well as tubular alignment, stability, and growth. Generally, the vesicles were 9:1 (by mass) zwitterionic-to-charged lipid ratio. However, this base ratio was altered to determine the effect of membrane properties on the onset voltage (the minimum field strength for tubule formation). Quantifying the effects is accomplished by noting the number of nanotubules per liposome versus composition and field strength. Also, the relative direction and linearity of the resulting tubules is noted versus field strength. A brief explanatory model of the effects based on lipid migration and local charge collection nucleating tubule growth is described.

Materials and Methods

Materials. L- α -Phosphatidylcholine (egg, chicken PC), L- α phosphatidic acid (egg, chicken-monosodium salt PA), and N-4-nitrobenz-2-oxa-1,3-diazole phosphatidic acid (NBD-PA) chloroform solutions and cholesterol powder (ovine wool > 98%) were obtained from Avanti Polar Lipids Inc. Oregon Green 488 1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine (Oregon Green DHPE) was obtained from Molecular Probes (Eugene, OR). Polybrene (PB) (>95%), potassium phosphate dibasic anhydrous, and sodium dihydrogen phosphate (99%) were obtained from Sigma-Aldrich Co. Nanopure 18 M Ω water was used for preparation of all solutions and is simply referred to as water here.

Preparation of Buffer Solutions. Phosphate buffer was made from prepared 10 mM solutions of sodium phosphate dibasic and sodium phosphate monobasic. A specific volume of phosphate monobasic was titrated with enough sodium phosphate dibasic until the desired pH of 7.4 was obtained.

Preparation of Liposomes. Liposomes were prepared by reverse-phase evaporation^{32,33} and consisted of various ratios of phosphatidylcholine, phosphatidic acid, and phosphoethanolamine with varying amounts of cholesterol. In brief, the chloroform-dissolved lipids were added to a round-bottom flask. While rotating the flask, the chloroform was rapidly evaporated off using nitrogen gas, leaving a thin, uniform solid gel coating of lipids on the interior of the flask. Remaining chloroform was removed by vacuum. The dry lipids were then prehydrated with a few microliters of water with rotation at 39 °C. Phosphate buffer was then added to the flask, which was allowed to rotate at 39 °C for 2 h. This method produces giant unilamellar and multilamellar vesicles.³⁴

Substrate Coating. Wells were formed, cleaned, and treated with a cationic surfactant according to methods developed for capillary electrophoresis surfaces treatments.³⁵ Wells were defined with silicon sealant (Permatex) to outline a rectangular well (capable of holding approximately 2 mL of solution) on a microscope glass slide (Figure 1A). The well was then rinsed with 10 mM NaOH for 5 min, then rinsed with water for 5 min, and gently patted dry with VWR light-duty tissue wipes (West Chester, PA). The well was then filled with 7.5% PB (w/v in water) for 15 min followed by a water rinse for 5 min.

Liposome Attachment. The liposome preparation was allowed to reside in the prepared glass slide well for 5 min. Phosphate buffer was then used to overflow the well with the purpose of displacing the nonattached liposomes from the remaining solution while preventing drying of the attachment surface. This procedure was performed until the remaining solution was visibly clear, indicating that most of the nonattached liposomes were removed from the surface. The outside of the well and the rest of the glass slide were

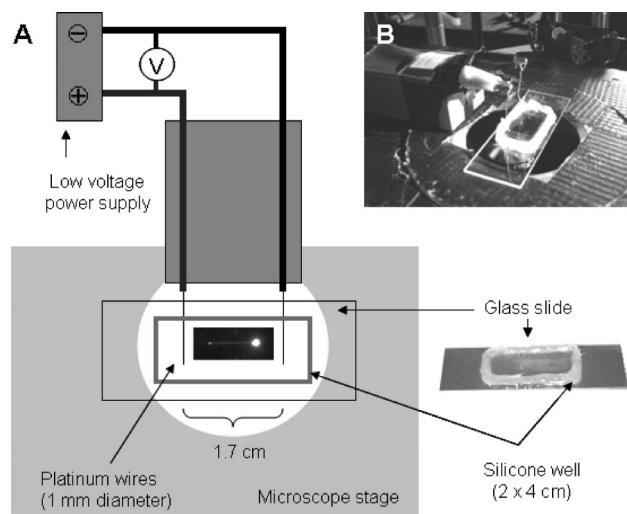


Figure 1. (A) Schematic (top view) of the experimental apparatus used for data collection along with a fluorescent image of a tubule growing from a liposome. The image illustrates the direction of tubular growth when an electric field is applied to surface-attached vesicles. (B) View of the experimental apparatus on the microscope stage during data collection.

completely dried with tissue wipes prior to beginning imaging with the microscope. Substrate coating procedures were performed at room temperature.

Preparation of Tubules. The apparatus shown in Figure 1A was assembled in house. It consisted of a simple circuit powered by a low-voltage power supply with a voltmeter attached in parallel. One centimeter sections of two platinum wires, 1-mm diameter and separated by a distance of 1.7 cm, were submerged in the sample well perpendicularly to the long axis of the microscope slide. Both electrodes rested on the substrate to ensure stability and submersion in the solution. Image collection was performed by fluorescence microscopy, starting with no electric field and progressively increasing the magnitude of the voltage applied.

Microscopic Observations. An inverted microscope with dark-field and fluorescence capability (IX70, Olympus) using a 100 W high-pressure Hg lamp as the light source was used to observe the liposome and tubular networks. Light from the mercury lamp was passed through a 460–500 nm band-pass filter and through a 40 \times objective to the sample. Emitted light was collected through a 505 nm long-pass dichromatic mirror and a 510–560 nm band-pass filter into the camera port on the microscope. Digital image collection was performed using a QICAM CCD camera (Q imaging Inc.) connected to a personal computer running Streampix III (Norpix).

Tubule Quantitation. A series of 10–20 images was captured at varying times during each applied voltage. The electric field was initiated or increased a few seconds before the first image at each value recorded. Images were generally taken around the midpoint area between the two electrodes. Each capture was manually quantified for the number of attached liposomes and number of tubules formed.

Results and Discussion

Lipid nanotubules were formed in a phosphate buffer solution on a microscope slide with fluorescently labeled lipids from mother liposomes (typical diameters of 10–50 μ m) immobilized on the glass substrate by electrostatic interaction. Modest electric fields (less than 20 V/cm) were applied to the surface-attached liposomes where well-behaved and stable growth of nanotubules was observed, even with fields as low as 2 V/cm. Previous work in our laboratory (unpublished results), indicates the elongated structures are lipid bilayer-bound tubules with an aqueous interior. Before the electric field is applied there is a relative absence of tubules and the majority of the objects observed are of spherical

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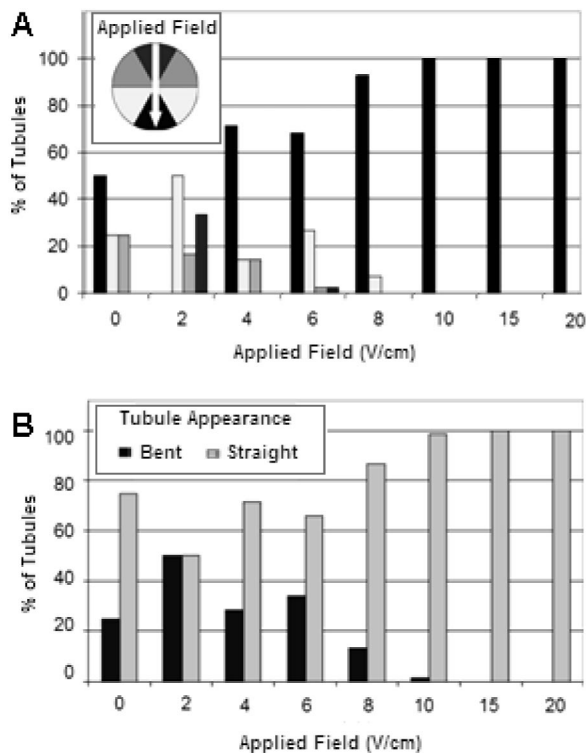


Figure 2. (A) Bar graph of tubule orientation vs applied field. The insert indicates the direction of the electric field, and the various regions represent the tube direction assuming the mother liposome is placed at the center of the circle. (B) Bar graph quantifying general tubule appearance vs applied field. In A and B data is presented as a percentage to account for the difference in abundance of tubular formation at various applied fields (e.g., when no field is applied elongated structures are scarce compared to when liposomes are exposed to higher magnitude fields). Liposome composition for both A and B was 89:10:1 PC:PA:OG-DHPE.

shape. Tubular structures begin to extend from the immobilized liposomes between a few seconds to a few minutes after the field is initiated. As the applied field increases, the system reaches a “tubule-saturation” point in which tubular growth is observed in most image frames.

Generally, the observed behaviors of tubular growth demonstrate that the orientation and stability of the lipid nanotubules is dependent on the magnitude of the applied field (Figure 2). At higher voltages, tubular formation occurs in the direction of the applied field. Conversely, at lower electric fields the orientation of tubular growth is more disperse and appears randomly. Tubules that grow at these low magnitude fields appear flexible and respond to local convective flow. As the magnitude of the applied field increases, the tubules appear less flexible and more stable (stability in terms of preserved geometric integrity). Images obtained at higher fields (generally higher than 8 V/cm) show lipid nanotubules mainly aligned parallel to each other with lengths of hundreds of micrometers at maximum extension.

Phospholipid tubules of different compositions exhibit diameters that range from nanometers to several micrometers (Figure 3). The larger tubular diameters (several microns) are more common during the initial stages of growth (Figure 3A). Smaller diameters (in the nanometer range) are exhibited for highly extended tubular structures (Figure 3B). However, the exact dimensions of smaller tubular inner and outer diameters are below the optical resolution and cannot be measured accurately with this technique. Nonetheless, previous studies in which tubules were obtained from liposomes (1–10 μm diameters and consisting of a 1:9 mixture of NBD-PA and PC) undergoing electrophoresis

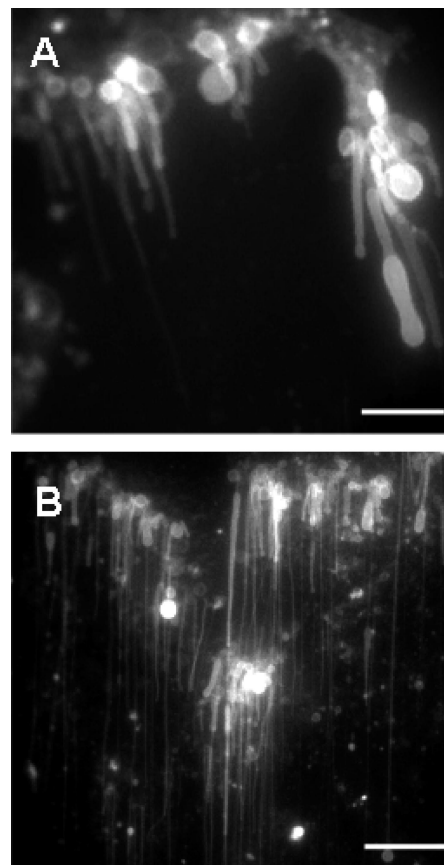


Figure 3. (A) Fluorescence micrograph of lipid nanotubules from a liposome composition of 89:10:1 PC:PA:OG-DHPE at an applied voltage of 8 V/cm. (B) Tubules from a liposome composition of 99:1 PC:OG-DHPE at 10 V/cm. Bar = 50 μm . The lipid tubules are generally aligned with the electric field.

have shown diameters of approximately 20 nm for tubular structures formed.²⁹ In this previous work liposomes were not surface immobilized and the applied field strength was greater than 40 V/cm (up to 330 V/cm). In addition to these previous observations theoretical calculations also suggest a submicrometer diameter for this type of tubular structure.²⁷

Qualitatively, the images collected showed a relatively well-ordered and stable growth pattern; however, this behavior is difficult to quantify. To quantitatively describe tubular growth the average number of tubules per liposome was determined from the images obtained at each applied voltage. A typical sigmoidal curve is obtained when plotting these values (Figure 4). Each data point represents the information obtained from a series of 10–15 fluorescence microscopy images from a particular experiment. As also seen qualitatively, the curve illustrates the relative absence of tubular structures at lower applied fields which changes steeply to a somewhat constant number of average tubules per liposome. This represents the stage in which tubule saturation takes place. Despite the optical and sequestrational limitation in our system (which can give rise to variability in the y axis of the plot and relatively large standard deviations), it is still possible to deduce a voltage range where tubular growth changes most dramatically. The behavior of tubular growth exhibited by different liposome systems suggests that there is an “onset voltage” that triggers tubular extension. This onset voltage represents a small range of magnitude of an applied field that provides sufficient force required to extend lipid nanotubules from the immobilized vesicles. To determine the variables that possibly influence the magnitude of the onset voltage, the mechanisms

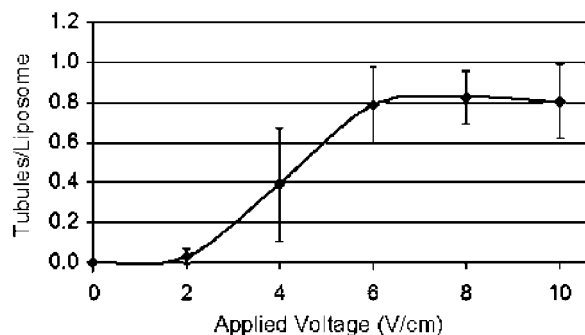


Figure 4. Plot of average tubule per liposome vs applied voltage for tubular formation in liposomes with a composition of 89:60:10 PC:PA:OG-DHPE by mass.

of tubular formation in our system as well as current theoretical models of tubular extension are examined.

The influence of an applied electric field on liposomes is complicated by the intrinsic field generated locally by the charged lipid headgroups of the bilayer.^{36–38} When a liposome is placed in a uniform electric field it distorts the electric field. The field exerts electric and mechanical stresses on the surface of the liposome that induce shape and topological changes as well as migration of individual lipids about the surface.³⁶ In moving to a small local deformation regime these shapes can be elongated into very slender bodies. This type of large transformation into slender bodies in the presence of electric fields has been confirmed both experimentally and theoretically with viscoelastic drop deformation in extensional flows.^{39–41} A similar principle can be applied to tubular formation from phospholipid vesicles as the resulting cylindrical shape represents a minimum in surface energy from a point force being applied to the liposome.

To determine the effect of different lipids on onset voltage, various vesicle compositions were examined. First, the charged lipid (PA) to zwitterionic lipid ratio (PC and OG-DHPE) and, second, cholesterol addition of various amounts were used to investigate the influence of the bending modulus parameter. This approach was chosen since cholesterol addition has been determined to be the single most effective way to increase the strength and modulus of a lipid bilayer.⁴²

The influence of varying the phosphatidic acid was investigated by forming nanotubules from mixed PC-PA-OG-DHPE vesicles at three different ratios: 89:10:1, 89:30:1, and 89:60:1 PC:PA:OG-DHPE (by mass). Quantitatively, the data trend suggests that increasing the amount of PA in the vesicles results in a lower average onset voltage for tubular formation (Figure 5). However, the error in these measurements suggests that these points could be consistent with a constant onset voltage, and further studies need to be completed in order to arrive at a more definite conclusion. Qualitatively, tubule saturation can be reached at a lower applied voltage as the percent PA increased. Less force is required to extend tubules from liposomes that have lower surface tension, meaning a lower magnitude electric field will trigger formation of nanotubules.

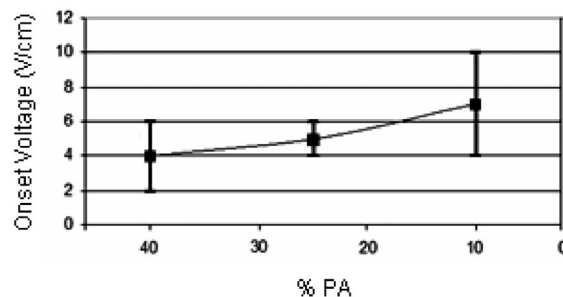


Figure 5. Onset voltage vs % PA for three different liposome compositions: 89:60:1, 89:30:1, and 89:10:1 PC:PA:OG-DHPE which correspond to 40%, 25%, and 10% (by mass), respectively.

The influence of cholesterol was investigated by forming nanotubules from liposomes (89:10:1 PC:PA:OG-DHPE, by mass) with various amounts of added cholesterol. The cholesterol percentages (by mass) used in this study were 0%, 8%, 15%, and 30%. Tubular growth is commonly observed in the absence of cholesterol (Figure 6B) at applied voltages as low as 5 V/cm. However, the average onset voltage increases as the percent cholesterol goes from 0% to 8% (Figure 6A). When the cholesterol content was 15%, only a few tubular formations were observed throughout the experiment. However, the number of tubules per liposome observed at this composition was not statically significant. At cholesterol compositions of 30%, only spherical structures were observed and no significant tubular structures were visible, even at fields of 25 V/cm (Figure 6C).

In terms of tubule initiation one possible mechanism is formation of a charged lipid domain in the surface of the liposome due to lipid migration as a consequence of the applied electric field. To briefly explore this models describe that the required force (f) to pull tubules is proportional to the square root of the bending rigidity (κ_c) and the surface tension (σ) according to^{6,27}

$$f = 2\pi(2\kappa_c\sigma)^{1/2} \quad (1)$$

This force has a characteristic value of a few piconewtons. The radius (R_t) of the nanotubules is the result of a force balance between membrane tension and curvature energy²⁸

$$R_t^2 = \kappa_c/2\sigma \quad (2)$$

For micromanipulation techniques used to form lipid nanotubules, the membrane tension of the mother liposomes can be controlled using micromanipulation protocols. However, our system does not involve direct mechanical intervention to form lipid tubules. Therefore, it is possible that these variables primarily depend on the composition of the immobilized vesicles. This is consistent with the observed dependence of the onset voltage value on liposome composition.

Assuming that a charged-lipid domain can be considered a point charge of charge (q), the electric force (f) experienced by the particle is the product of the electric field (E) wherein the particle is located and the particle charge. Applying this mathematical relation ($F = Eq$) suggests that the amount of charge that can provide enough force to extend nanotubules is reasonable in terms of both the electric field magnitude used in this experiments and the size of the charged “patch” that would need to form on the surface of the vesicle. The required force, a few piconewtons, is being provided (tubules form) using an average electric field of 5 V/cm (for noncholesterol experiments). The total charge required by this electric field magnitude to produce a force of approximately 10 pN will have a characteristic value of 10^{-14} C. Assuming an average number of charges per

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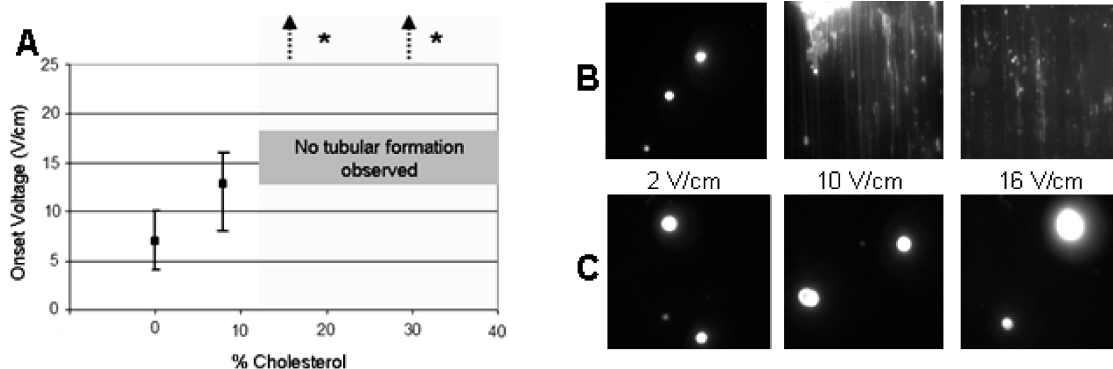


Figure 6. (A) Onset voltage vs applied field for tubule formation with various concentrations of cholesterol in a mixed PC:PA:OG-DHPE liposome (89:10:1 PC:PA:OG-DHPE, wt %). Onset voltage increases as % cholesterol increases; however, no tubular formation was observed at higher (>8%) cholesterol amounts using electric fields as high as 25 V/cm. (B) Dark field microscopy images of liposomes/tubules at 0% cholesterol and (C) 30% cholesterol under applied voltages of 2, 10, and 16 V/cm.

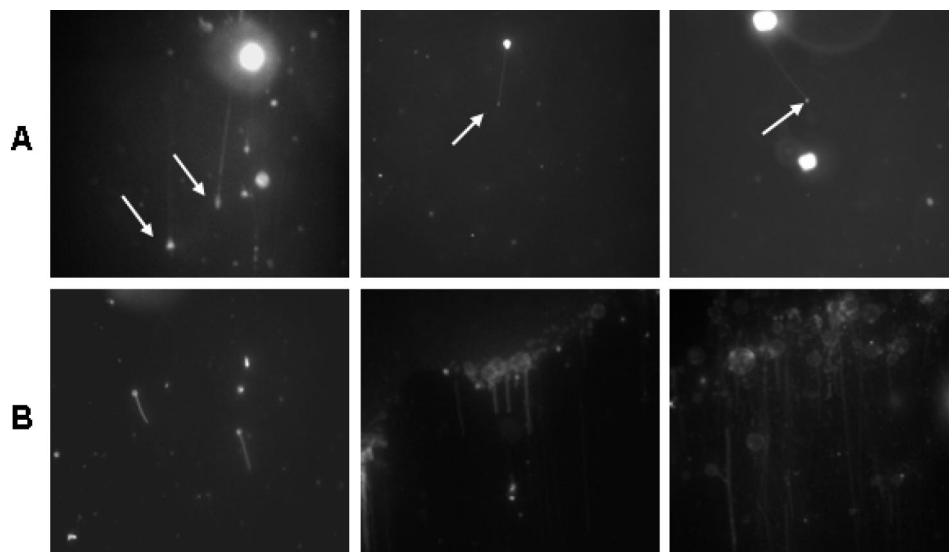


Figure 7. Fluorescence microscopy images of liposomes and growing tubules labeled with (A) NBD-PA, showing the brighter tips most likely due to a collection of labeled lipids, and (B) OG-DHPE

lead domain and taking in consideration the cross-sectional area of a phospholipid head group, this total charge will correspond to an initial lipid domain of roughly 50 nm radius in the vesicle surface. A lipid “patch” of these dimensions can reasonably be present in vesicles with radial dimensions in the microscale, similar to the ones in our experiments.

Detailed observation of lipid domains in the surface of the liposomes is difficult in our experiments because their dimensions are likely below the optical resolution. Conversely, formation of small domains in the resulting tubules has been commonly observed when the liposomes composition used contained labeled charged lipids. While certainly not optically resolved in detail, the spatial distribution of charged lipids at the tip and along the tubule is readily observed in experiments in which liposome composition was 89:10:1 PC:PA:NBD-PA (by mass). For tubules labeled with NBD-PA, the growth tip appears much brighter than the body of the tubule, suggesting formation of a small domain of the labeled, charged head groups (Figure 7). In experiments where NBD-PA was used, growth of this general type was by far most commonly observed once a tubule was initiated.

The absence of tubular structures at high cholesterol content was not unexpected. Roux and co-workers provided evidence of

the mechanisms of lipid sorting in membrane tubes; they reported that cholesterol appears to be critical for formation of lipid domain by favoring lipid mixing, thus influencing the lipid sorting process.^{43,44} One of the mechanisms described involves an initial phase of mixed lipids in the membrane which subsequently becomes a system of sorted lipids upon tubular formation on the basis of the molecular properties of the individual lipids.⁴³ As discussed, a possible mechanism for tubular formation using electric fields is that the charged lipids migrate within the surface of the liposome and form a leading domain that initiates extension of the tubules from the mother liposome surface.³⁶ If the presence of cholesterol hinders lipid segregation, a leading domain becomes more difficult to form at high cholesterol concentrations. The lack of a significant amount of lipid tubules at high cholesterol content (more than 15%) suggests that cholesterol addition hinders or delays a possible lipid sorting mechanism that precedes tubular extension.

The work presented here shows that dramatic changes in the shape of lipid bilayers in surface-attached liposomes result from electric fields of much lower strength than used in previous studies (50–300 V/cm) in which liposomes were imaged while

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undergoing electrophoresis in fused silica capillaries.²⁹ The liposome immobilization approach taken in this current study not only seems to reduce the magnitude of the applied field needed for the formation of tubules but also provides the possibility to tailor the location of the liposomes within the surface of attachment for future phases of this research.

Our approach for lipid tubular formation is simple and opens exciting opportunities for fabrication of these structures for both application and fundamental contexts. Understanding that tethered bilayer-bound systems can be directly controlled by an external electric field suggests entirely new and complex networked structures can be created by choosing the charge state of the liposome and providing surface attachment with addressable molecular recognition elements, such as cDNA.^{45,46} The magnitude and direction of the electric field can be chosen such that interconnecting networks of nanoscale membrane-bound system can be created without direct mechanical intervention.⁶

There are, of course, several important issues to be addressed. Further attention to kinetics of tubular growth in relation to magnitude of field applied and liposome composition is still needed. Variables such as liposomes size, temperature, and adhesion chemistry and their influence on tubular formation using electric fields need to be further understood. In addition, a more

thorough theoretical description needs to be developed to depict our method of tubular formation. These new approaches would closely examine the disturbance that the field creates on the membrane which consequently leads to lipid migration, conglomeration, and extension events.

Conclusion

This approach demonstrates that lipid nanotubules can be extended from surfaced-attached liposomes using low-magnitude electric fields (less than 20 V/cm), showing that lipid tubules of mixed-lipid vesicles can be prepared without direct mechanical manipulation. Tubules from several micrometers to even millimeters in length can grow simultaneously from a mother vesicle. Tubule alignment can potentially be controlled by varying the direction of the applied field. The composition of the liposomes can affect the overall onset voltage for tubular formation, and adding cholesterol can hinder tubule formation. This model increases the bending modulus of the bilayer, while higher ratios of phosphatidic acid suggest a decrease in the overall onset voltage for tubule growth by possibly decreasing the liposomes' surface tension.

Acknowledgment. This work was funded, in part, by Arizona Applied NanoSensors.

LA8028897

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