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Scanning Electrochemical Microscopy. 56. Probing Outside and Inside Single Giant Liposomes Containing $Ru(bpy)_3^{2+}$

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Giant liposomes containing $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) were prepared as model systems for biomembranes and cells and studied by scanning electrochemical microscopy (SECM). Conical carbon fiber tips of submicrometer size were used to approach, image, and puncture individual liposomes immobilized on glass substrates. SECM images of the liposomes were obtained, and the leakage of $Ru(bpy)_3^{2+}$ through the lipid membrane was probed. The tip was also pushed into liposomes and characteristic breakthrough transients, corresponding to liposomes with different compartmental configurations, were obtained. Voltammograms were obtained with the tip inside a single liposome after breaking through the membrane, and the influx of mediator and efflux of encapsulant after puncture could be observed.

Giant liposomes encapsulating redox molecules were prepared and probed by scanning electrochemical microscopy (SECM). Conical carbon fiber electrodes of a submicrometer tip size were employed to approach, image, and puncture individual liposomes. Such studies are of interest in characterizing the liposomes but also in using them as models for biological cells. Characteristic breakthrough (puncture) current transients corresponded to liposomes of different compartmental configurations, and voltammetric curves with the tip inside the liposome could be obtained. The leakage of the encapsulated redox molecules from liposomes was also studied by positioning electrodes close to (<200 nm) the surface of a single liposome.

Liposomes of various sizes and configurations have been made and studied, for example, as biomembrane and protocell models.[1,2] Liposomes have an improved structural stability over bilayer lipid membranes (BLMs),[3] because their formation generates a closed assembly of lipid molecules and thus a more stable stand-alone system. Giant liposomes, with diameters of a few micrometers and above, are particularly attractive systems because of their accessibility to optical microscopy and various micromanipulation techniques.[4−6]

Liposomes have also been pursued as biocompatible carriers and containers in biomedical and pharmaceutical applications. Indeed, many hydrophilic compounds, including drugs, enzymes, and DNA, have been successfully encapsulated in liposomes.[4,7] Of interest in such systems is the rate at which materials leak out of the liposome. By combining redox-encapsulated giant liposomes with SECM techniques, one should be able to detect the diffusional efflux of encapsulated molecules from individual liposomes. Such efflux can result from either a natural leakage process or membrane structural changes during and after a biochemical event. Pores or channels can also be incorporated into liposome membranes to control movement of species across the membrane. Nanometer-sized liposomes containing redox species have been prepared as electrochemical tags for amplified biochemical detection and sensing[8,9] and are being studied in our laboratory as labels for electrogenerated chemiluminescence (ECL). We show here that SECM[10] allows one to probe individual giant liposomes and obtain useful information about biomembranes in general.

SECM is a scanning probe technique in which electrochemical signals can be measured with high spatial resolution.[10] Instrumentally, SECM comprises an ultramicroelectrode as a tip, a high-precision position controller, and a potentiostat. With a redox mediator present in the system, positive or negative feedback approach curves can be generated when the tip is brought close to a conducting or insulating substrate surface. These curves can then be fit to theoretical models to obtain the tip-to-substrate distance and then used to study electrochemical processes near the interface. A significant advantage of SECM is that it is capable of probing electrochemical processes with fast kinetics, since the steady-state measurements largely remove signal perturbations due to charging and adsorption. Recently, SECM has been applied in a number of cellular studies,[11−14] most of which involved using

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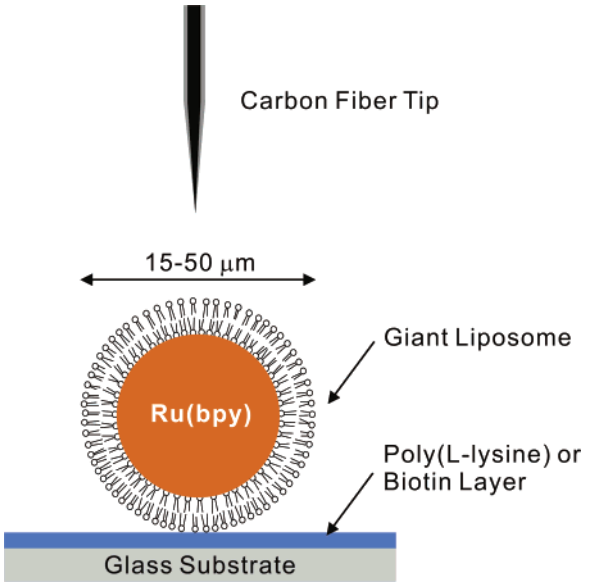

Figure 1. Experimental setup. Liposomes of 15−50 μm were immobilized on glass substrates via either poly(L-lysine) or biotin−avidin−biotin sandwich structure; these liposomes were probed by a submicrometer-sized carbon fiber tip controlled by the SECM.

microelectrodes positioned outside cells to monitor the transport of electrochemically active species across cell membranes, e.g., caused by cellular respiration or uptake and expulsion of species by cells. It would also be of interest to monitor redox processes and respiration directly *inside* biological cells. Considering the architectural and functional complexity of such cells, we thought it desirable to start with an analogous system of a simpler configuration where the structure and chemical content can be fully controlled. The concept of moving a SECM tip from a solution phase into a polymer phase was demonstrated previously, e.g., in a study of the penetration of a Nafion membrane and the observation of electrochemistry of a species incorporated in the polymer film.[15] In this paper, as shown in Figure 1, we used SECM to probe immobilized liposomes to obtain information about their behavior.

EXPERIMENTAL SECTION

Materials and Methods. Chemicals. Several lipids, including 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DSPG), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol) (sodium salt) (SOPG), 1,2-dimyrisoyl-*sn*-glycerophosphocholine (DMPC), and 1,2-dimyrisoyl-*sn*-glycero-3-[phosphor-rac-(1-glycerol)] (sodium salt) (DMPG), all with purity above 99%, were obtained from Avanti Polar Lipids, Ltd. (Alabaster, AL). *N*-(6-(Biotinoylamino)hexanoyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (sodium salt) (biotin-X-DSPE, purity >95%) was purchased from Northern Lipids Inc. (Vancouver, BC, Canada), Cholesterol (purity >99%) was from Aldrich. EZ-Link Sulfo-NHS-LC−Biotin (sulfosuccinimidyl-6-(biotin-amido)-hexanoate) and FITC conjugated NeuraAvidin were obtained from Pierce Biotechnology, Inc. (Rockford, IL) The SECM mediator, ferrocenylmethyltrimethylammonium ($FeCp_2TMA^+$) perchlorate, was prepared through metathesis by using the corresponding iodide salt (Strem Chemicals, 99%) and silver perchlorate (Aldrich, 99.9%). Ruthenium(III) hexamine chloride (99%) was also from Strem Chemicals (Newburyport, MA).

Preparation of $Ru(bpy)_3^{2+}$ Encapsulated Giant Liposomes. The preparation of $Ru(bpy)_3^{2+}$ encapsulated giant liposomes generally followed the previously reported double-emulsion technique[16−18] with certain modifications detailed in the following. In a 1.4 × 4.5 cm glass vial (Teflon-lined screw cap), 8 μmol of DSPC with either 2 μmol of DSPG or 0.5 μmol of SOPG was first dissolved in 1 mL of chloroform/ethyl ether (1:1, v/v). In this lipid solution, 0.5 mL of 50 mM $Ru(bpy)_3Cl_2 \cdot 6H_2O$ (Aldrich) dissolved in a 0.15 M sucrose (Sigma) aqueous solution was then added. A water (containing $Ru(bpy)_3^{2+}$)-in-oil emulsion was then formed by vortexing the capped mixture for 15 s. The resulting emulsion was layered on 3 mL of a 0.2 M sucrose aqueous solution in a 50-mL round-bottom flask. Immediately after this step, the flask was transferred to a water bath at 65−70 °C and the organic phase (chloroform and ethyl ether) was removed by passing argon into the flask with gentle swirling for 5 min. The flask was then capped with a glass stopper and kept in the same bath for 55 min. A similar procedure was followed to prepare biotin-containing liposomes, in which case 2% biotin-X-DSPE was codissolved with the other lipids in the chloroform/ethyl ether mixture. To coat the biotinylated liposomes with avidin, these liposomes were incubated in an excess amount of 0.2 mg/mL NeutrAvidin (Pierce) for 1 h and separated from the unreacted medium by centrifugation.

The liposomes were separated from untrapped materials and lipid debris by centrifugation (Centrifuge 5415D, Eppendorf) at 800*g* for 5 min using Tris-HCl buffer saline (10 mM Tris and 0.1 M NaCl, pH 7.0) as the suspension medium.

Liposome Immobilization on Glass Substrates. Two methods were used to immobilize giant liposomes on glass slides. In both methods, microscope slides (Erie Scientific, No. $1^1/_2$ thickness) were first cleaned with dilute detergent solution (Micro-90, International Products, Burlington, NJ) by sonication for 30 min. The slides were then rinsed thoroughly with deionized water (Milli-Q reagent water system, Bedford, MA) and sonicated in methanol for another 30 min. These slides were further rinsed with deionized water, dried in a 110 °C oven, and finally sandwiched between a Teflon electrochemical cell and the microscope stage ring (see below).

In the first method, the negatively charged liposomes were immobilized on the glass surface coated with poly(L-lysine). Briefly, the glass slides were first treated with 0.01% (w/v) poly(L-lysine) (Sigma) for 30 min. After being rinsed with water and dried with argon, the slides were incubated in a 200-μL aliquot of liposome dispersion (in Tris-HCl buffer, 10 mM Tris and 0.1 M NaCl, pH 7.0) for 30 min. Because the liposomes contained ~200 mM encapsulated sucrose, they tended to settle on the glass and thus interact with the positively charged poly(L-lysine)-coated glass slides. The remaining suspended liposomes were then removed

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from the electrochemical cell by replacing the suspension solution with 10× volume of Tris-HCl buffer.

In the second approach, the liposomes containing 2% biotinylated lipid were anchored on the glass surface using a biotin−avidin−biotin sandwich structure.[19] Biotin groups were linked to glass surface using the following two-step procedure. In the first step, the clean and dry glass slides were soaked in 2% (v/v, in acetone) (3-aminopropyl)triethoxysilane (Sigma) with sonication for 1 h. After rinsing with water and drying in the oven (110 °C), the slides were treated with 5 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in phosphate buffer solution (0.1 M, pH 8.0) for 30 min. The unreacted agent was then removed, and for the liposome immobilization, the biotinylated glass slides were incubated with 200 μL of avidin-coated liposome (in Tris-HCl buffer) at 25 °C for 1 h. The remaining suspended liposomes were removed by flushing the electrochemical cell with 10× volume of Tris-HCl buffer.

Electrochemistry. A commercial SECM (model 900, CH Instruments, Austin, TX) was used to control the tip potentials, obtain the approach curves, and monitor the tip-to-substrate and tip-to-liposome distance. To quickly target individual liposomes immobilized on glass substrate, the SECM micropositioning head was mounted on the stage of an optical/fluorescence microscope (Eclipse TE300 Nikon inverted microscope, Melville, NY). A color CCD camera (Magnafire S99806, Olympus) was used to obtain optical and fluorescence images.

The microtip electrodes used in the SECM measurement were made from flame-etched carbon fibers[20] partially insulated with anodic electrophoretic paint,[21,22] as described below. First, a ∼1-cm segment of carbon fiber (Goodfellow, 10-μm diameter) was glued onto a Nichrome wire (Alfa Aesar, Ni/Cr 80:20 wt %, 250-mm diameter) with silver epoxy. This assembly was then inserted into a borosilicate glass pipet (Sutter Instrument, o.d. 1.0 mm, i.d. 0.58 mm) previously pulled by a laser pipet puller (model P-2000, Sutter Instrument). A one-line program was used in the pulling with the parameters heat 350, filament 4, velocity 30, delay 100, and pull 0, which generated a pipet tip with an inner diameter of ∼20 μm. Next, the two ends of the glass pipet were sealed directly with the Nichrome wire and carbon fiber, respectively, by melting the glass with an oxygenated gas flame. The pulled tip side, especially, was sealed in such a way that a ∼2-mm-long carbon fiber extended from the glass tip. The exposed carbon fiber was further shortened and sharpened by the same flame so that the apex of the resultant carbon fiber tip generally had a diameter less than 1 μm as determined by an optical microscope. Finally, the exposed carbon fiber electrodes were coated with anodic electrophoretic paint (Glassphor ZQ 84-3211, BASF). In this step, the carbon fiber was immersed in the 1:20 diluted paint solution in deionized water and a bias of 2.2 V was applied between the fiber and a coiled Pt wire. The paint was deposited onto the fiber under a potential bias for 30 s, the carbon fiber tip was transferred into an oven (150 °C), and the paint was cured for 3 min. The paint deposition and thermal treatment was repeated once, and the tips were then ready for SECM experiments.

The SECM experiments were performed in a homemade Teflon electrochemical cell with a conventional three-electrode configuration. The counter and reference electrode was a Pt wire and silver wire quasi reference electrode (precoated with AgCl), respectively. The bottom of the cell was the microscope glass slide (No. 11/2 thickness) sandwiched between the microscope stage ring and the Teflon cell via rubber O-rings. A fiber-optic lamp and a homemade clear-acrylic tip holder were employed to facilitate tip positioning over immobilized liposomes.

RESULTS AND DISCUSSION

$Ru(bpy)_3^{2+}$ Complex Encapsulation in Giant Liposomes. There are several methods available in the literature for giant liposome preparation. Among these, the electroformation method developed by Angelova and Demitrov[23−25] is the most widely used because it allows the ready preparation of large (i.e., several hundred micrometers) and often unilamellar liposomes. Although the exact formation mechanism is still unclear, the charged headgroups in the lipids used likely play a determining role in the process. For this reason, this method only works well when little or no other electrolytes are present. Under these conditions, the alternating field applied can act directly on the lipid itself; the electric field is largely shielded when a large amount of electrolyte is present. We could prepare giant liposomes by elecroformation when water was used as the solvent, but when 5 mM $Ru(bpy)_3^{2+}$ (in deionized water) was added, essentially no liposome formation was observed. Two other methods, based on either solvent evaporation[26] or self-growth from dried lipids,[27] did produce some giant liposomes; however, the as-prepared liposomes always contained a large portion of other lipid structures, e.g., clumps and tubes.

Stable, $Ru(bpy)_3^{2+}$ encapsulated giant liposomes were prepared by the double-emulsion method[16−18] as described in the Experimental Section. With this method, 50 mM $Ru(bpy)_3^{2+}$ complex dissolved in an aqueous 150 mM sucrose solution was first entrapped in an inverted micelle of lipids (i.e., water-in-oil (w/o) emulsion) by vortex shaking. This emulsion mixture was then introduced into an aqueous buffer, resulting in a water-in-oil-in-water (w/o/w) system. Upon removal of organic (oil) phase by heating and purging with inert gas, the lipids initially dissolved in the oil phase tended to "collapse" on those lipids surrounding the inner aqueous core, and as a result, liposomes were formed. Coulter counting results indicated most of liposomes prepared were smaller than 10 μm with a size distribution peak at ∼2 μm (data not shown). However, it was not difficult to find larger liposomes, i.e., with diameters of 30−50 μm, to study by SECM, since larger structures are easier to work with. Optical and fluorescent microscope images of thus prepared liposomes resuspended in Tris-HCl buffer (10 mM Tris and 0.1 M NaCl, pH 7.0) are shown in Figure 2A and B.

Liposomes are generally good carriers for compounds, either hydrophilic or hydrophobic, in which lipid bilayers can act

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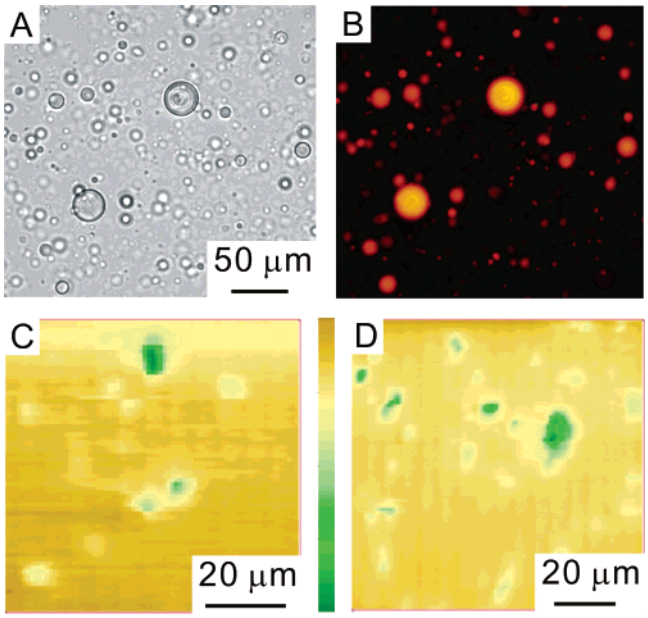

Figure 2. Preparation of $Ru(bpy)_3^{2+}$ encapsulated giant liposomes. (A) Optical image of liposomes of DSPC/DSPG/biotin-X-DSPE (molar ratio, 80/20/2) prepared by the double-emulsion method (see text). Liposomes were pelleted by centrifugation and resuspended in Tris-HCl buffer (10 mM Tris, 0.1 M NaCl, pH 7.0). (B) The corresponding fluorescence micrograph of (A). The orange fluorescence is from $Ru(bpy)_3^{2+}$ encapsulated inside liposomes ($\lambda_{ex} = 490$ and $\lambda_{em} = 520$). (C) SECM image of liposomes of DSPC/DSPG/biotin-X-DSPE (molar ratio, 80/20/2) immobilized on poly(L-lysine)-coated glass slides; scale, 75 × 75 μm. Tips were biased at −0.4 V vs Ag QRE. Current range (as shown by the color scale from green to brown), (3.2−4.0) × 10^{-10} A. Scale bar in (A) also applies to pictures in (B). (D) SECM images of immobilized liposomes comprising DSPC/cholesterol/DSPG (molar ratio, 4.5/4.5/1). Tips were biased at −0.4 V vs Ag QRE. Current range, (4.6−5.9) × 10^{-10} A. Solution for SECM imaging, 1.0 mM ruthenium(III) hexamine in 100 mM phosphate buffer, pH 7.0.

effectively as a physicochemical barrier to stop these compounds from penetration.[1] In this sense, $Ru(bpy)_3Cl_2 \cdot 6H_2O$ is not a good candidate for encapsulation, because it has fairly high solubility in both water and organic solvents. We used this compound in our redox encapsulation experiments, however, because of its analytical importance and high quantum efficiency in ECL.[28,29] In addition, the fatty acid chain length and unsaturated conditions of the lipid determine the gel-to-liquid-crystalline phase transition temperature (Tc), which in turn, affects the membrane fluidity and thus permeability of the encapsulants.[1] Indeed, in our experiment, we found $Ru(bpy)_3^{2+}$ could be successfully entrapped in DSPC/DSPG (both with Tc = 55 °C) liposomes but not when the SOPC (Tc = −20 °C)/SOPG (Tc = −18 °C) pair was used instead. This illustrates the strong effect of lipid structure on liposome properties, because the only difference between these two pairs of lipids is the latter has a double bond in one of the acyl chains. We also tested the DMPC/DMPG (saturated chains, Tc = 23 °C) pair using a similar approach. Here the liposomes first formed successfully but they underwent deformation after being stored at 4 °C overnight. While phosphatidylcholine was the major component in our preparation, we found it necessary to include a certain amount of phosphatidylglycerol to prevent

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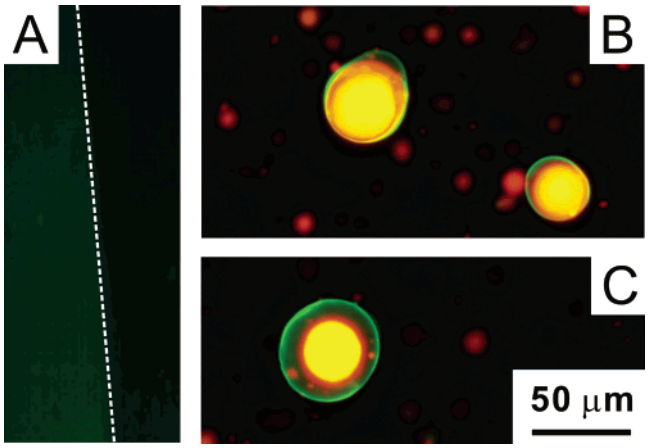

Figure 3. Steps to immobilize liposomes on glass slides via biotin−avidin−biotin sandwich linkage. (A) Biotinylation of glass slides. Shown here is a fluorescence image of a biotinylated glass slide after treatment with avidin−FITC for 30 min. The right side of the dashed line has not been biotinylated (but similarly silanized and treated with avidin−FITC). The contrast of the image was decreased to suppress the fluorescence from nonspecifically adsorbed avidin−FITC. (B) Fluorescence micrograph of $Ru(bpy)_3^{2+}$ encapsulated liposomes (DSPC/SOPG/biotin-X-DSPE (molar ratio, 80/5/2) coated with avidin−FITC ($\lambda_{ex} = 490$ and $\lambda_{em} = 520$). (C) Another fluorescence micrograph of $Ru(bpy)_3^{2+}$ encapsulated liposomes after being coated with avidin−FITC. This heterogeneous liposome consists of a $Ru(bpy)_3^{2+}$-rich core and a lumen filled with aqueous medium.

the liposome deformation when they were resuspended in electrolyte solutions. Similarly, we excluded cholesterol in our method, because liposomes containing cholesterol underwent irregular shape changes when exposed to electrolyte solutions, as shown in Figure 2D imaged by SECM. However, these cholesterol-containing liposomes could keep their spherical shape when resuspended in an aqueous 5% (w/v) glucose solution instead.[17]

Liposome Surface Immobilization. Glass pipets with a diameter of a few hundred nanometers have been successfully pushed inside electroformed giant liposomes[30,31] In these experiments, the pipets were controlled by a micromanipulator and the liposomes were pushed against a solid support, i.e., a Pt wire or a microfence structure. In our case, the microtip electrodes were lowered perpendicularly toward a flat substrate, on which liposomes were preimmobilized. It thus became important to ensure that the immobilization was sufficiently strong to withstand any perturbations by the tip. Since most of the liposomes we prepared are multilamellar,[18] it is possible to tie them down to glass surface with either a poly(L-lysine) or a biotin−avidin linkage without rupturing the lipid.[31] While the method based on poly(L-lysine) is quicker to implement, because it involves fewer steps, the biotin−avidin−biotin sandwich structure offers a stronger link between liposome and glass substrate and therefore was better when the tip was used to break through the membrane to probe the liposome interior. Figure 3 records the interaction between biotin and avidin, on either glass or liposome surface, as indicated by FITC−avidin fluorescence. Interestingly, it also shows liposomes of different structure, i.e., a single core across the liposome

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versus a core surrounded by lipid bilayer(s) of larger diameter, can be formed in the same batch. As will be shown later, these two configurations generate different current transients as the tips breaks into the liposomes.

Carbon Fiber Tip Characterization. Conventional SECM measurements generally employ microdisk electrodes because the techniques to fabricate these are well established and the electrochemical behavior can be readily characterized by voltammetry. However, to break through the lipid bilayers easily, conical electrodes with a high aspect ratio and small RG (relative thickness of the insulating sheath) value are preferred. We thus developed carbon fiber microtip electrodes by first fabricating nanoneedle carbon fiber electrodes using flame etching[20] and then insulating the carbon fiber with anodic electrophoretic paint[21,22] (see Experimental Section). Using an oxygenated gas flame to etch carbon fiber of 10-μm diameter, we could routinely make carbon fiber electrodes with the tip apex size smaller than 500 nm. An experimental approach curve over an insulating substrate is shown in Figure 4A. To find the zero tip−substrate separation ($d = 0$) for the approach curves with these small conical tips, care must be taken not to hit the substrate, since even a light touch causes a change in $i_{T,\infty}$. One can determine the $d = 0$ point over an insulator by moving the tip down until i_T just drops below $i_{T,\infty}$. From there the tip is lowered in small increments and then raised to remeasure $i_{T,\infty}$ until an increase is noted. For these tips, which could be constructed quite reproducibly, this occurred when $i_T \approx 0.8\ i_{T,\infty}$. This curve was fit to the theoretical model for SECM with a conical shape tip,[32] yielding a base radius of 0.4 μm, an RG = 1.2, and an aspect ratio (H) of ~3. Approach over a gold substrate using the same tip showed positive feedback curves but was slightly below the line with the above parameters (Figure 4B). The deviation observed with the positive feedback approach curve suggests an even larger value of H, probably 4−5. Conical tips with large aspect ratios are not useful for measuring approach curves with good sensitivity, because the contribution from electrochemical processes at the side of the cone make a large contribution compared to that from feedback, so simulations with such large aspect ratios have not been carried out. A cyclic voltammogram (inset, Figure 4A) of the mediator ($FeCp_2TMA^+$) oxidation taken with the tip in bulk solutions shows the typical sigmoidal-shape response characteristic of a microelectrode.[33]

SECM Imaging of Immobilized Liposomes. The immobilized liposomes could be imaged by SECM. An approach curve in a solution of 1.0 mM ruthenium(III) hexamine in 100 mM phosphate buffer, pH 7.0, was first taken over the glass substrate. The tip was then stopped at the position where the current had decreased to 80% of $i_{T,\infty}$ and then retracted a distance of 15 μm. An $x-y$ scan at this long distance was then carried out and the current recorded did not show good contrast. The tip was brought down at 2-μm steps in z-axis until features in the images were obtained. Typical SECM images of the immobilized liposomes on glass are shown in Figure 2C and D.

SECM Study of $Ru(bpy)_3^{2+}$ Leakage. We attempted to probe the leakage of $Ru(bpy)_3^{2+}$ from individual liposomes by placing a tip near the liposome and monitoring the current for $Ru(bpy)_3^{2+}$ oxidation. In this experiment, the carbon fiber tip was first lowered to within 200 nm from the immobilized liposome surface by recording the negative feedback approach curves of 0.7 mM $FeCp_2TMA^+$ acting as the mediator. The mediator solution was then removed from the electrochemical cell and replaced by fresh buffer, and a 1.2 V potential was then immediately applied to the tip to detect any $Ru(bpy)_3^{2+}$ that flowed from the liposome. However, the current transient in this position was essentially the same as that when the tip was far from the liposome in pure buffer solution. This very low leakage of $Ru(bpy)_3^{2+}$ from intact liposomes was confirmed in electrogenerated chemiluminescence experiments that will be reported elsewhere.

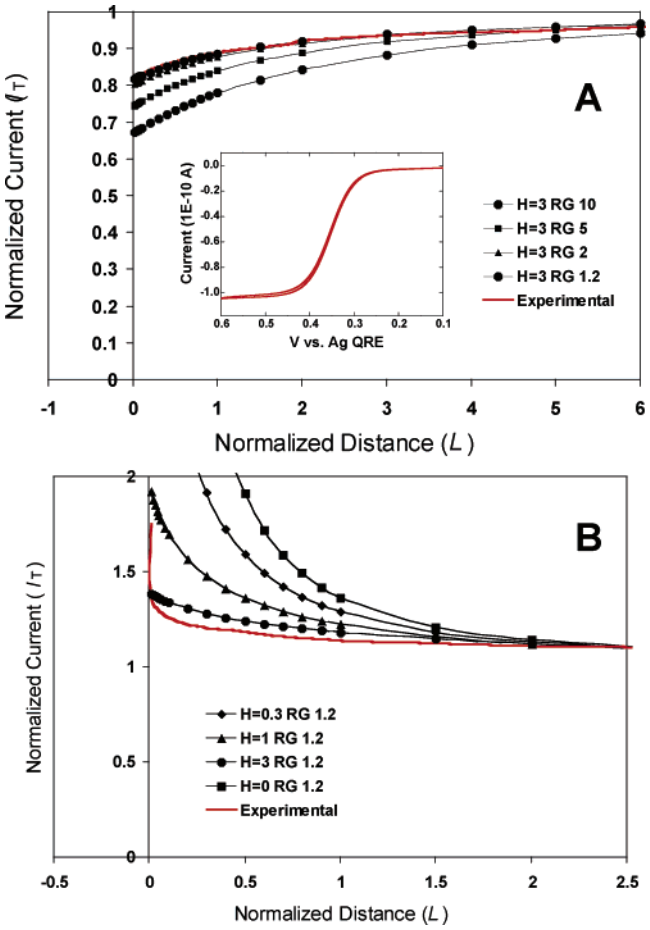

Figure 4. Comparison of the experimental SECM approach curves (red lines) to theoretical ones (black lines) over different substrates or liposomes. The experimental curves were obtained using carbon fiber tips (see text) in a 0.7 mM ferrocenylmethyltrimethylammonium perchlorate buffer solution (Tris-HCl, 10 mM Tris and 0.1 M NaCl, pH 7.0). The tip electrode was biased at 0.5 V vs Ag QRE and traveled down at a maximum distance increase of 50 nm. (A) Approach curves over an insulator. Inset: a typical CV response obtained with a carbon fiber microtip electrode. (B) Approach curves over a conductor. The tip and substrate electrode was biased at 0.5 and 0 V vs Ag QRE, respectively.

Tip Approach and Breakthrough of Liposomes. The potential of using giant unilamellar vesicles (GUVs) as a model for biological cells has long been recognized, and a great deal of research has been done with GUVs as microreactors, membrane protein carriers, and prototype cells.[4] However, there have been few attempts of using GUVs as the basic platform to study ion channels and other pore-forming membrane proteins. One reason for this has been the technical difficulty associated with GUV preparation. Electroformation is only capable of producing giant

(32) Zoski, C. G.; Liu, B.; Bard, A. J. *Anal. Chem.* **2004**, *76*, 3646−3654.

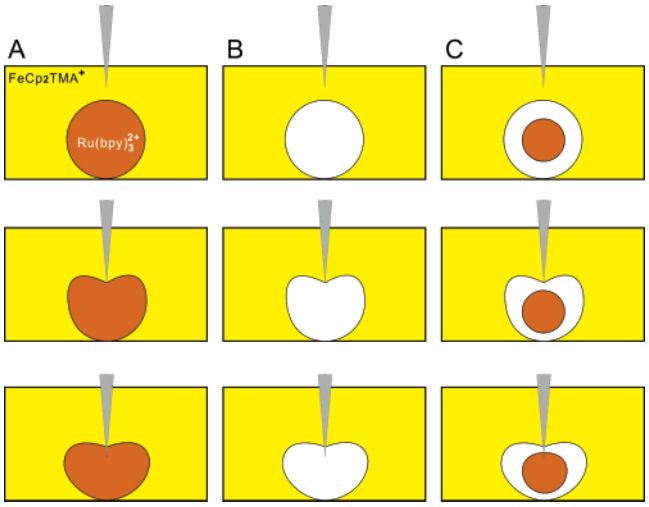

Figure 5. Schematic presentation of different liposomes that undergo shape deformation during the course of tip breakthrough experiments (from top to bottom). Liposomes are divided into three kinds based on their $Ru(bpy)_3^{2+}$ content and distribution.

liposomes under low ionic strength, which seriously limits its usefulness under physiological conditions. Another related problem is the low sealing stability of micropipet-impaled liposomes, which is a required configuration in general electrophysiological measurements across membranes.[31]

Fluorescence microscopic results revealed the double-emulsion method we used produced mainly three types of giant liposomes. While most of these liposomes had $Ru(bpy)_3^{2+}$ distributed homogeneously inside the lumen, a small portion contained a $Ru(bpy)_3^{2+}$-rich core (small liposomes) surrounded by a buffer-rich lumen (Figure 3). In addition, a still smaller number contained no $Ru(bpy)_3^{2+}$ inside. Similar to the wide size distribution of the liposomes we prepared, the content variation can be also attributed to inhomogeneous emulsion formation/solvent evaporation processes. We thus expect to see different electrochemical behavior when liposomes of different configuration are approached and punctured by a carbon fiber tip, as schematically shown in Figure 5. Moreover, there is the question of tip modification by membrane components as the tip passes into the liposome.

In doing these experiments, immobilized liposomes of different compartmental configuration were first selected (by comparing optical and fluorescence images) and the approach curves toward the glass substrate were then made with the aid of a mediator in the solution. The tip was then pulled back about 20−30 μm (depending on the liposome size) and, by optical microscopic observation, moved over a targeted liposome; approach curves directly above the lipid membrane were then obtained. To break through into the immobilized liposome, an approach curve was first obtained in which the tip was stopped when the current dropped to 85% of the steady-state tip current in the bulk ($i_{T,\infty}$). This normally brought the tip down to less than 200 nm above the membrane surface (as estimated from the finite-element analysis model for conical tips,[32,34] see Figure 4). A new approach was then started at this point (i = 85% $i_{T,\infty}$) while the potential

(33) Zoski, C. G. *Electroanalysis* **2002**, *14*, 1041−1051.

(34) Note that higher tip aspect ratio (>3) means the electrode apex is brought even closer to the liposome surface as compared to a tip of H = 3, when the same level of current decrease was monitored.

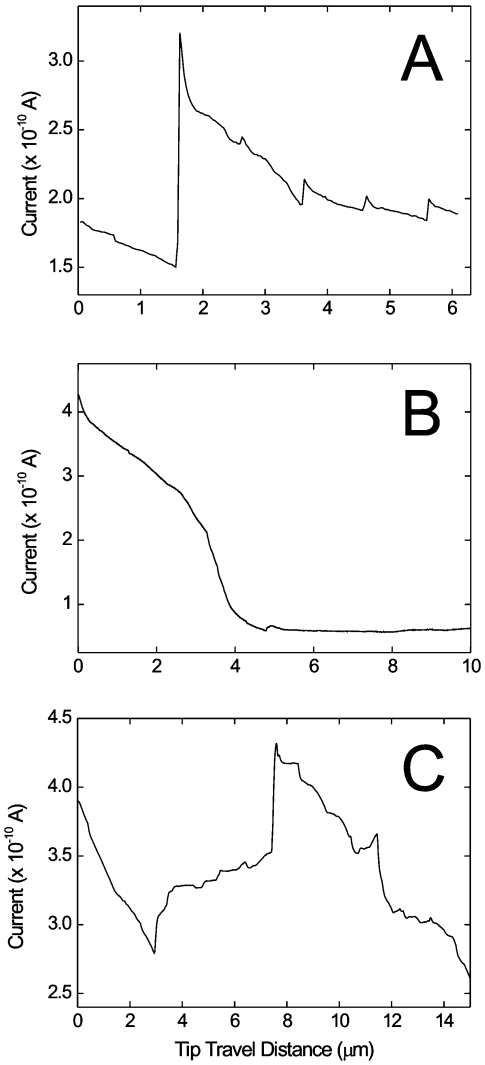

Figure 6. SECM monitoring of tip current when it breaks through the bilayer(s) of different liposomes. (A−C) correspond to the three different situations outlined in Figure 6. In this series of experiments, the tip potential was held at 1.2 V (vs Ag QRE) where $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$. The regular, 1-μm-apart current spikes in the approach curves are due to the mechanical clicks of the inchworm motor used. A 1 mM ferrocenylmethyltrimethylammonium perchlorate ($FeCp_2TMA^+$) in Tris-HCl buffer (10 mM Tris and 0.1 M NaCl, pH 7.0) was used as the redox mediator.

was increased to 1.2 V (vs Ag QRE) where $Ru(bpy)_3^{2+}$ is oxidized. As shown in Figure 6, different characteristic breakthrough curves were observed, consistent with the different cases shown schematically in Figure 5. In all the three cases, a decrease of current was first observed as the tip traveled down to the surface. After the apex of the tip touched the membrane, a further lowering of tip deformed the lipid bilayers downward, accompanied by a further drop of the current. This effect is reminiscent of the SECM behavior noted when a tip approaches the interface between an aqueous solution and an immiscible liquid and a thin layer of solution is trapped.[35] The breakthrough of the liposome normally took place a few micrometers below the point where the tip first touched the liposome surface, which varied from one experiment to another due to differences in the tip sharpness and liposome

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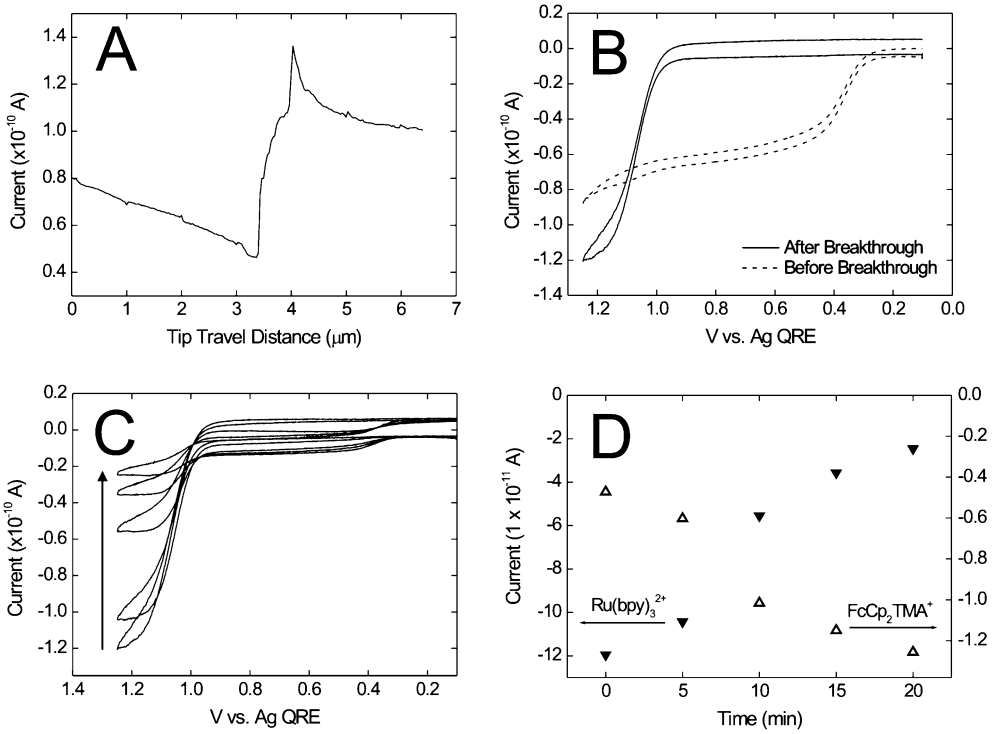

Figure 7. Voltammetric responses of $Ru(bpy)_3^{2+}$ confined in a single giant liposome. (A) Current response of a carbon fiber tip undergoing liposome breakthrough, in which the tip potential was held at 1.2 V (vs Ag QRE). (B) Comparison of cyclic voltammograms taken when tip was outside and inside the liposome. The potential was scanned from 0.1 to 1.25 V (vs Ag QRE) where oxidization of both the mediator ($FeCp_2TMA^+$) and $Ru(bpy)_3^{2+}$ occurred. Potential scan rate, 50 mV/s. (C) Liposome exchange of content with surrounding medium upon breakthrough by a tip. The encapsulated $Ru(bpy)_3^{2+}$ leaked out of the liposome, as indicated by a decrease of the oxidation current along the arrow direction. Each CV was taken ~5 min after the previous one. (D) The current decrease of $Ru(bpy)_3^{2+}$ oxidation over time was accompanied by an increase of $FeCp_2TMA^+$ oxidation.

size (thus the extent of bending deformation of the membrane before breakthrough).[36] At the breakthrough point, a transient with a sharply rising current was obtained for a liposome (Figure 6A). This represents the sudden immersion of the tip into a solution of $Ru(bpy)_3^{2+}$ and a potential step (Cottrell current) transient along with a contribution from charging current. For a liposome that did not contain electroactive species, $Ru(bpy)_3^{2+}$, a decrease of current was observed on breakthrough (Figure 6B). The small steady-state current observed in this case represents the background current in the electrolyte with perhaps a very small contribution from mediator that leaked into the liposome during breakthrough. Similarly, a rise of current was only observed following a decrease in current (Figure 6C) when the tip broke into the core of a multilamellar liposome whose core was filled with $Ru(bpy)_3^{2+}$, (as shown in Figure 5C). While the exact approach curves varied from one experiment to another, the same general trend was essentially followed. Additional cases of microtip breaking in $Ru(bpy)_3^{2+}$ encapsulated liposomes are shown in the Supporting Information.

A more conclusive result is shown in Figure 7, where cyclic voltammograms (CVs) were recorded while the tip was held inside a liposome a few micrometers below the breakthrough point. Here, the potential was scanned between 0.1 and 1.25 V, where the oxidation of both the mediator ($FeCp_2TMA^+$) and $Ru(bpy)_3^{2+}$ were covered. A scan before breakthrough (dashed line, Figure 7B) shows only mediator oxidation. The first scan after breakthrough (solid line, Figure 7B) shows only the oxidation wave of $Ru(bpy)_3^{2+}$, indicating the tip was indeed inside the liposome and the membrane had completely blocked the entry of $FeCp_2TMA^+$ into the liposome at this time. Four more consecutive scans were then made at 5-min intervals (Figure 7C). The limiting current for $Ru(bpy)_3^{2+}$ oxidation decreased over time, while the limiting current for $FeCp_2TMA^+$ oxidation increased. This result indicates that the sealing of lipid layer around a microelectrode tip impaled inside the liposome is not leak-free, and as a result, species can diffuse in and out of the liposome driven by the concentration gradients across the membrane. As time elapsed, the redox species started to equilibrate across the lipid membrane and the current leveled off (Figure 7D). If 20 min (time between the breakthrough and the finish of the fifth CV) is long enough for $FeCp_2TMA^+$ to equilibrate across the membrane and taking the diffusion coefficients of $Ru(bpy)_3^{2+}$ and $FeCp_2TMA^+$ as 6×10^{-6} and 7.5×10^{-6} cm^2 s^{-1}, respectively, we estimate that the initial concentration of $Ru(bpy)_3^{2+}$ inside the liposome was ~15 mM. This value is lower than the initial concentration of $Ru(bpy)_3^{2+}$ in the solution used for liposome preparation, presumably, due to the leakage of the encapsulant during the various dilution/separation steps in the experiment. By comparing the observed current magnitude for $FeCp_2TMA^+$ oxidation in Figure 7B and C, the surface of the carbon fiber electrode seems to be significantly modified by lipids during the breakthrough. The details of this modification are currently not clear and will be investigated further.

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CONCLUSIONS

We explored the possible usefulness of SECM in the study of liposomes, with potential applications to biomembranes and redox regulation of cellular processes. We demonstrated individual redox encapsulated giant liposomes could be probed by microelectrode tips to obtain useful information about molecular transport through a biomembrane. This adds an alternative probe to those based on fluorescence and radioactivity in studying biomembranes. In comparison to a BLM setup[37,38] we previously used to study charge and ion transfer through bilayer lipid, the present system based on giant liposomes allows one to perform measurements over a more extended period of time. With a stable biomembrane system in hand, we anticipate carrying out more detailed studies on the liposome membrane modified by natural or synthetic proteins as pores and channels at higher resolution.

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SUPPORTING INFORMATION AVAILABLE

Additional figures are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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