Reversible Vesicle Restraint in Response to Spatiotemporally Controlled Electrical Signals: A Bridge between Electrical and Chemical Signaling Modes†

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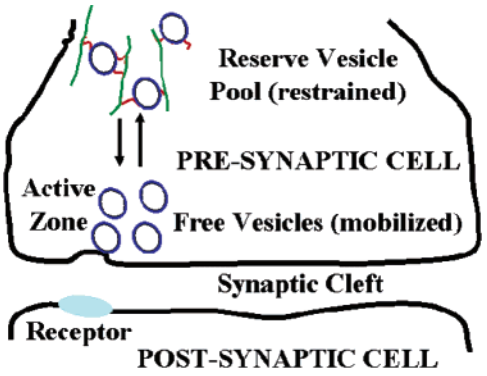
Microelectronic devices employ electrons for signaling whereas the nervous system signals using ions and chemicals. Bridging these signaling differences would benefit applications that range from biosensing to neuroprosthetics. Here, we report the use of localized electrical signals to perform an operation common to chemical signaling in the nervous system. Specifically, we employ electrical signals to restrain vesicles reversibly. We perform this operation using the stimuli-responsive aminopolysaccharide chitosan that is able to electrodeposit onto cathode surfaces in response to localized electrical stimuli. We show that surfactant−vesicles and liposomes can be co-deposited with chitosan and are entrapped (i.e., restrained) within the deposited film's matrix. Vesicle co-deposition could be controlled spatially and temporally using microfabricated wafers with independent electrode addresses. Finally, we show that vesicles restrained within the deposited chitosan matrix can be mobilized under mildly acidic conditions (pH <6.5) that resolubilize chitosan. Potentially, the ability to restrain and mobilize chemical signals that are segregated within vesicles may allow microfluidic systems to access the rich diversity offered by chemical signaling.

Introduction

The signal processing capabilities of microelectronic devices are often compared to those of the nervous system, yet their differences are profound.[1] Microelectronic devices convert input into output through intermediate steps involving the flow of electrons, and the nervous system converts input to output using ions and molecules. Although comparing the brain and computer is instructive, bridging the signaling differences could offer substantial practical benefit. For instance, the effective interfacing of microelectronic devices with biology could provide new opportunities for applications that range from biosensing to neuroprosthetics. Here, we report the use of a localized electrical signal to perform a chemical signaling operation common in the nervous system.

Chemical signaling is largely irrelevant for conventional microelectronic devices because they function under dry conditions and require only spatiotemporal control of electron flow. However, the opportunity/need to control chemical signaling becomes considerably greater as microfabrication is extended to microfluidic devices.[2] In contrast to an electronic device that uses a single signaling species (the electron), fluids can contain a multitude of signals. For instance, a nerve cell can simultaneously (or nearly simultaneously) use Na^+, K^+, and Ca^{2+} as ionic signals and at the same time transmit and receive information using a range of chemical species (neurotransmitters). Thus, biology may provide important lessons on how to segregate and control individual chemical signals within fluidic environments.

Scheme 1



Neurotransmitters are important signaling molecules that mediate communication between cells in the nervous system, and biology employs various mechanisms to segregate, transport, store, transmit, and receive neurotransmitters. As illustrated in Scheme 1, neurotransmitter molecules are released at the synapse by "upstream" presynaptic cells, and these chemical signals are received by receptors in "downstream" postsynaptic cells. Historically, downstream responses were observed to be quantized, and this quantization was correlated to upstream structures in the presynaptic cell. Specifically, presynaptic cells store thousands of neurotransmitter molecules in vesicles that can fuse with the cell membrane to release their contents into the synaptic cleft. Importantly, the presynaptic cell can possess a reserve pool of vesicles that are restrained by association with the cytoskeleton (i.e., actin filaments). The presynaptic cell recruits these restrained vesicles by an intracellular signaling pathway

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that is only partially understood but is known to involve protein phosphorylation.[3−7] Biology routinely utilizes protein phosphorylation/dephosphorylation for intracellular signaling. Unfortunately, the complexity of these intracellular signaling cascades makes them inconvenient mechanisms to reproduce in cell-free applications. Here, we employ localized electrical signals as a more convenient means to restrain vesicles.

For vesicle restraint, we employ the pH-responsive and film-forming aminopolysaccharide chitosan to recognize localized cathodic signals and deposit as a stable thin film.[8,9] Mechanistically, chitosan's electrodeposition occurs because electrochemical reactions at the cathode generate a pH gradient, chitosan chains that experience the high localized pH adjacent to the cathode become deprotonated and insoluble, and these chains deposit as a hydrogel film.[10−14] Once neutralized, the deposited chitosan film is stable in the absence of an applied voltage. However, chitosan's deposition is reversible in the sense that reexposure of the film to acidic conditions (pH less than ∼6.5) can reprotonate chitosan's amines and resolubilize the polysaccharide. Importantly, several groups have shown that nanoscale components can be co-deposited with chitosan and entrapped within the electrodeposited chitosan network.[14−22] Here, we extend these observations to demonstrate that chitosan's electrodeposition can be used to co-deposit and restrain vesicles, whereas resolubilization of the deposit can mobilize the previously restrained vesicles.

Materials and Methods

The following chemicals were purchased from Sigma-Aldrich Chemicals: chitosan from crab shells (85% deacetylation with a molar mass of 370 000 reported by the supplier); cetyl trimethylammonium tosylate (CTAT); sodium dodecylbenzene sulfonate (SDBS); 5(6)-carboxyfluorescein (CF); sulforhodamine 101 (RA); tris(hydroxymethyl)aminomethane (Tris); 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES); and chitosanase (specific activity of 205 U/mg). CF-labeled chitosan was prepared by reacting chitosan with NHS-fluorescein as previously described.[11] L-α-Phosphatidylcholine (PC) from egg and ammonium 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (RA-PE) were obtained from Avanti Polar Lipids, Inc. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Invitrogen.

Surfactant vesicles and liposomes were prepared as previously described,[23] and no effort was made to control the internal pH or salt content. Surfactant vesicles were prepared by mixing the cationic surfactant CTAT and the anionic surfactant SDBS in a 70:30 mass ratio in distilled−deionized water and gently stirring for 2 days.[24] These catanionic vesicles have been observed to be stable even in high salt environments.[25] If the vesicles were to contain a water-soluble fluorescent dye, then we included either CF (1 mM) or RA (0.5 mM) in the initial CTAT and SDBS mixture. To purify dye-containing vesicles from "free" dye in the mixture, we used a Sephadex G-50 column. Liposomes were prepared by an extrusion procedure recommended by the manufacturer (Avanti Polar Lipids, Inc.). CF-containing liposomes were prepared in deionized water from PC (20 mM) and CF (15 mM) and purified of free CF using a Sephadex G-50 column. Labeled liposomes were prepared in deionized water from PC (13 mM) and either DiI (13 μM) or RA-PE (1.3 μM).

Gold-coated "chips" (either patterned or unpatterned) were fabricated from silicon wafers using standard methods.[11] Electrodeposition was performed by negatively biasing the chip while it was partially immersed in a solution containing both chitosan (1 w/w%) and vesicles. A dc power supply (mode 6614C, Agilent Technologies) was used to supply a constant current to the chip and counter electrode during deposition. After deposition, the electrodes were disconnected from the power supply, and the chips were rinsed several times with distilled water. In some experiments, electrodeposited films were peeled from the chips for analysis.

Films that had been peeled from the wafer were examined using a luminescence spectrometer (PerkinElmer Instruments LS55) to determine the fluorescence emission spectra. For this analysis, the excitation wavelength was 493 nm, and the emission was scanned from 500 to 600 nm. Films were also examined using a laser scanning confocal microscope (Leica TCS SP2) with a 100× oil-immersion objective lens (pinhole diameter 0.182 mm, Airy unit of 0.999216, and *z* resolution of about 200 nm). To image the CF-containing vesicles, we used an excitation wavelength of 488 nm and collected the emitted light in the range of 510−540 nm.

Photographs of the chips were taken using a digital camera (Canon EOS D-60) with a 90 mm lens. The photomicrographs of fluorescent chips were taken from a fluorescence stereomicroscope (MZFLIII, Leica) equipped with a digital camera (Spot 32, Diagnostic Instruments). To observe CF fluorescence, the microscope was set with an excitation wavelength of 480 nm (bandwidth of 40 nm) and a long-pass emission filter at 510 nm. To observe RA and DiI fluorescence, the filters were chosen with an excitation wavelength of 560 nm (bandwidth of 40 nm) and an emission filter at 610 nm. In all cases, ImageJ 1.34S, from NIH, was used to analyze images and quantify the fluorescence intensity.

Dynamic light scattering (DLS) measurements were used to determine vesicle size distributions. All measurements were performed using a Photocor-FC light-scattering instrument with a 5 mW laser light source at 633 nm with a scattering angle of 90°. A logarithmic correlator was used to measure the intensity autocorrelation function. Hydrodynamic radius distributions were extracted from the correlation functions using the Dyna-LS software package supplied by Photocor.

Results and Discussion

Vesicle Restraint by Co-deposition with Chitosan. To provide initial evidence for the co-deposition of vesicles with

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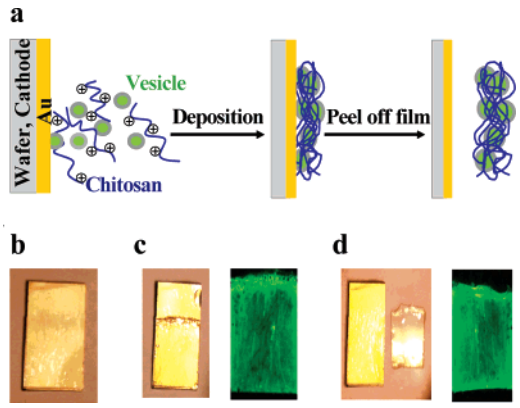

Figure 1. Initial evidence for co-deposition of CF-containing surfactant vesicles with chitosan. (a) Schematic of experiment. (b) Photograph of a gold-coated chip. (c) Photograph (left) and fluorescence photomicrograph (right) of a chip with an electrodeposited film. (d) Photograph (left) and fluorescence photomicrograph (right) of a film after peeling it from the wafer.

chitosan, we performed the experiment outlined in Figure 1a. For this experiment, we prepared a solution of chitosan and carboxyfluorscein (CF)-containing surfactant vesicles. The gold-coated wafer (8 × 8 mm^2) of Figure 1b was partially immersed in this solution and negatively biased to 16 A/m^2 for 10 min. After deposition, the power supply was disconnected, and the wafer was rinsed several times with distilled water. The photograph in Figure 1c shows that chitosan deposits onto the portion of the wafer that had been immersed in the deposition bath and the deposit adheres to the gold surface. The fluorescence photomicrograph in Figure 1c shows that the deposited film is fluorescent, which indicates that CF is present in the deposit. As indicated in Figure 1d, the deposited chitosan film can be peeled from the gold-coated wafer, and this film is also fluorescent. The results in Figure 1 provide initial evidence for the co-deposition of vesicles with chitosan.

We performed three additional experiments to provide independent evidence that intact vesicles are co-deposited with chitosan. First, we compared the loss of fluorescence upon washing between films prepared with CF-containing vesicles and control films prepared with free CF. For the control, we co-deposited from a solution containing chitosan with free CF. (There were no vesicles in this control.) After deposition at 16 A/m^2 for 10 min, the film was peeled from the wafer and washed multiple times with HEPES buffer (10 mM, pH 8). Figure 2a shows that the intensity of the emission spectra for this control film decreases continuously with these sequential washes. By the sixth wash, only limited CF emission is observed in this control film. This observation suggests that free CF can be substantially washed from the electrodeposited network. A second film was prepared by electrodeposition from a solution containing chitosan and CF that was present in both free and vesicle-bound forms. After depositing and peeling, we washed this experimental film as described above. Figure 2b shows a substantial reduction in fluorescence emission intensity after the first couple of washes; however, the emission intensity remained relatively constant after the third wash. The difference between parts a and b of Figure 2 suggests that intact, CF-containing vesicles are restrained within the deposited chitosan network.

In a second experiment, we separated CF-containing vesicles from free CF and co-deposited these CF-containing vesicles with chitosan (16 A/m^2 for 10 min). After deposition, the film was rinsed with distilled water, peeled from the wafer, and examined using a confocal microscope. Figure 3 shows the optically sectioned fluorescence images in the direction normal to the

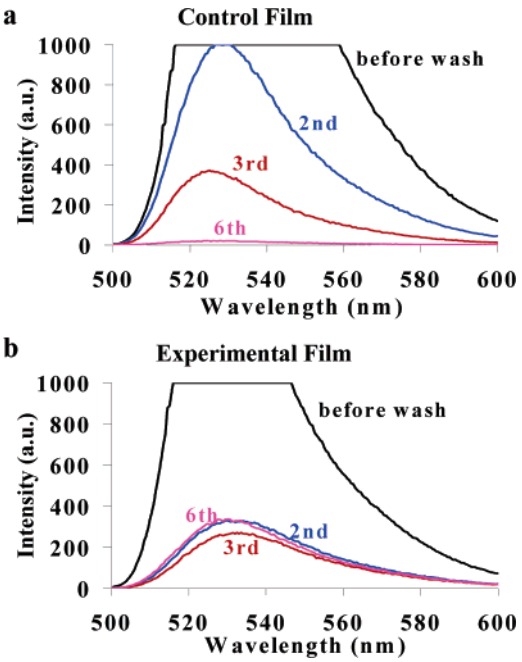

Figure 2. Evidence that CF-containing surfactant vesicles can be restrained within the electrodeposited chitosan matrix. (a) Fluorescence spectra of the control film prepared by co-depositing free CF with chitosan and subjecting the film to multiple washes with HEPES buffer (pH 8). (b) Fluorescence spectra of the experimental film prepared by co-depositing CF-containing vesicles with chitosan and washing multiple times. The retention of fluorescence in part b suggests that vesicles are retained within the deposited matrix.

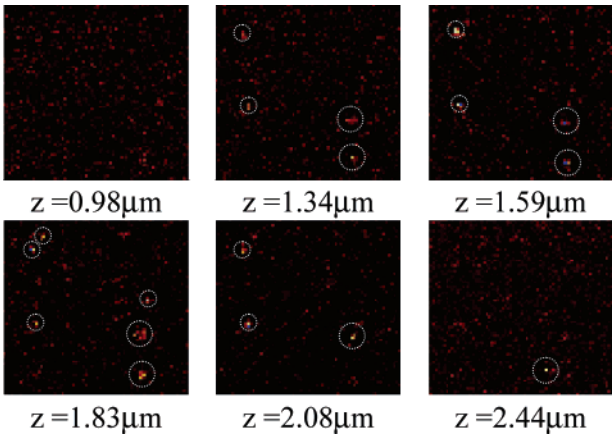

Figure 3. Evidence from confocal laser microscopy that CF-containing surfactant vesicles are intact within the electrodeposited film. A series of images from optical sections indicate that fluorescence is associated with finite particles (e.g., vesicles). Each image represents a 20 × 20 μm^2 area, and the fluorescent particles are circled in each image.

substrate surface (i.e., the *z* direction). As shown, a small number of fluorescent "particles" are observed in most fields of view. (These particles are circled in Figure 3.) Moreover, these fluorescent particles are confined to specific *z* positions, although the resolution is insufficient to determine the particle's size or whether the particles are isolated vesicles or vesicle aggregates. In a control experiment in which free CF was co-deposited with chitosan, we observed no fluorescent particles in the optical sections (images not shown). Thus, Figure 3 provides independent evidence that intact CF-containing vesicles are present within the electrodeposited chitosan matrix.

In a third experiment, we used dynamic light scattering (DLS) to provide evidence that intact vesicles are electrodeposited. Figure

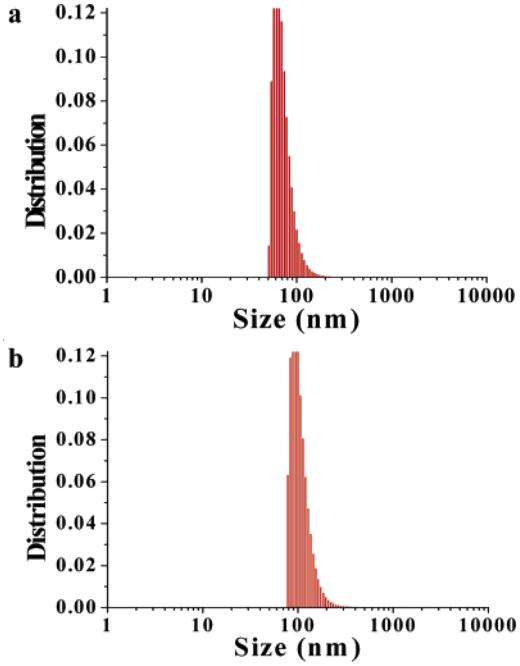

Figure 4. Evidence from dynamic light scattering that intact surfactant vesicles can be recovered from the deposited films. (a) Particle size distribution for initial surfactant vesicles (before mixing with chitosan). (b) Particle size distribution for vesicles recovered from an electrodeposited film by acid treatment (to dissolve the chitosan matrix) and chitosanase treatment (to cleave the polymer and reduce the sample viscosity).

4a shows the particle size distribution for the original surfactant vesicle solution. These vesicles were then mixed with chitosan and co-deposited (16 A/m^2 for 10 min). After deposition, the film was peeled from the wafer and rinsed with distilled water to remove surface-bound vesicles. The chitosan film was then resolubilized using an acetic acid solution (0.2 M acetate, pH 5), the chitosan chains were hydrolyzed with the enzyme chitosanase (0.1 U/mL), and the resulting solution was analyzed by DLS. Figure 4b shows the particle size distribution of this resulting sample. Although these measurements do not provide quantitative information on the fraction of intact vesicles, they do indicate that the size distribution of the released particles is similar to the distribution observed for the starting surfactant vesicles.

In summary, a combination of independent experimental observations supports the conclusion that intact vesicles are co-deposited with chitosan and restrained within the film network. Individually, none of these observations constitutes proof that the vesicles are intact; however, we believe that together these observations provide a weight-of-evidence that supports this conclusion.

Spatial and Temporal Control of Vesicle Restraint. To demonstrate that locally applied electrical signals can be used to restrain (i.e., co-deposit) vesicles, we fabricated the "chip" in Figure 5a. This chip has two electrically independent 1-mm-wide gold bands separated by a 1-mm-wide space. During deposition, the chip is partially immersed in solution such that the bands (i.e., the electrodes) are submerged while the leads remain above the liquid level and can be connected to the dc power supply. Initially, the chip was immersed in a solution of chitosan plus CF-containing vesicles, and the right-most electrode was negatively biased to 35 A/m^2 for 30 s. (The left electrode was unbiased during the initial deposition step.) After washing this chip, green fluorescence was observed on the right electrode as shown by the fluorescence photomicrograph in Figure 5b. This chip was next immersed in a solution of chitosan plus

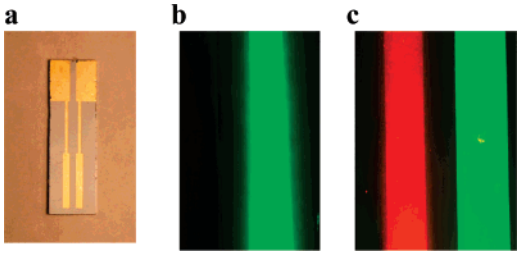

Figure 5. Spatiotemporal control of surfactant vesicle co-deposition. (a) Photograph showing the patterned chip with two independent 1-mm-wide electrode addresses. (b) Fluorescence photomicrographs after deposition of the CF-containing vesicles (green) on the right electrode. (The left electrode was unbiased during the first deposition step.) (c) Fluorescence photomicrographs after deposition of RA-containing vesicles (red) on the left electrode. (The right electrode was unbiased during the second deposition step.)

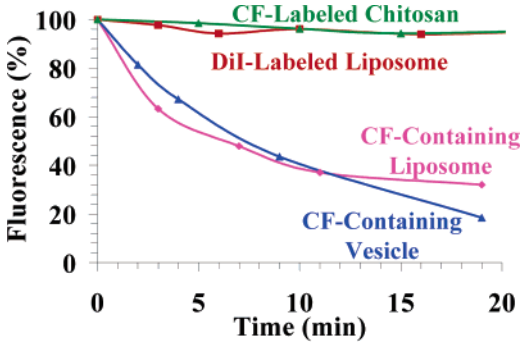

Figure 6. Leakage of fluorescence from electrodeposited films. When CF-containing vesicles or liposomes are co-deposited with chitosan, fluorescence is observed to leak from the deposited matrix. When CF is covalently grafted to chitosan and the CF-chitosan conjugate is deposited, no fluorescence leakage is observed. When a fluorescently labeled (DiI-labeled) liposome is co-deposited with chitosan, no fluorescence leakage is observed.

sulforhodamine (RA)-containing vesicles, and the left-most electrode was negatively biased to 60 A/m^2 for 15 s (the right electrode was unbiased during the second deposition step). [A higher voltage and shorter time were used during this second deposition step in an effort to limit the observed losses of green fluorescence from the previously deposited film on the right electrode. (See below.)] Figure 5c shows that red fluorescence is observed on the left electrode after this second deposition step whereas green fluorescence is retained on the right electrode. Moreover, both bands are well separated from each other. The results in Figure 5 demonstrate that vesicles can be deposited at separate electrode addresses with spatiotemporal control based on where and when voltage is applied.[26]

Although the results of Figure 5 indicate that vesicles can be restrained by electrodeposition, we persistently observed leakage of CF fluorescence from the deposited films. This leakage is illustrated by experiments in which CF-containing surfactant vesicles or CF-containing liposomes were co-deposited with chitosan (35 A/m^2 for 1 min). After deposition, each chip was immersed in 5 mL of distilled water, and the fluorescence intensity of the deposit was monitored over time using fluorescence microscopy. Figure 6 shows the loss in fluorescence intensity for films containing either CF-containing vesicles or liposomes.

One possible explanation for this loss of CF is the slow erosion of the electrodeposited chitosan. To test this possibility, we covalently grafted CF to the chitosan backbone and electro-

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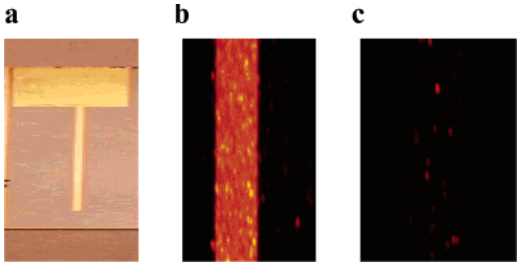

Figure 7. Evidence for liposome mobilization by acid treatment to dissolve the chitosan matrix. (a) Photograph showing the chip with a 200-μm-wide electrode used for co-deposition. (b) Fluorescence photomicrograph after co-deposition of DiI-labeled liposomes with chitosan. (c) Fluorescence photomicrograph after exposing the co-deposit to pH 6 buffer for 10 min. The loss of fluorescence suggests liposome mobilization.

deposited the CF-labeled chitosan (35 A/m^2 for 1 min). Figure 6 shows no loss in fluorescence from deposits of CF-labeled chitosan, indicating that there is little erosion of chitosan from the deposit. A second possible explanation for the observed leakage of CF is that the co-deposited vesicles or liposomes are being destroyed and releasing their contents over a 20 min time course. To test this second possibility, we co-deposited DiI-labeled liposomes (35 A/m^2 for 1 min). Figure 6 shows no loss of fluorescence from chitosan films with DiI-labeled liposomes over the 20 min time course shown. (In fact, no losses were observed even after 3 h). Because the DiI label is embedded within the liposome's bilayer, the constant fluorescence in this film suggests that the electrodeposited vesicles are not lost by destruction of the bilayer structure. After discounting our first two possibilities, we can offer two additional possible explanations for the observed loss of fluorescence from films with CF-containing vesicles or liposomes. First, the water-soluble CF may leak from inside intact vesicles/liposomes and then diffuse from the chitosan matrix. Second, a "bolus" of CF may exit from the vesicles/liposomes into the chitosan matrix during the deposition process, and then this free CF leaches from the matrix. Such a sudden release of CF into the matrix during deposition could occur if some fraction of the vesicles/liposomes are damaged or destroyed during deposition. Alternatively, vesicles/liposomes may be prone to sudden leakage during deposition because interactions between vesicles and polymers often lead to substantial structural changes in vesicle size[27] and shape.[28−30] For instance, we previously observed that interactions between vesicles and a hydrophobically modified chitosan led to a 3-fold reduction in vesicle diameter (from 120 to 40 nm).[24,31]

In summary, localized electrical signals can be employed to co-deposit vesicles at specific electrode addresses with spatial and temporal control. Further studies will be required, however, to characterize the structure and stability of these restrained vesicles.

Vesicle Mobilization. We next performed experiments to demonstrate that vesicle restraint can be reversed and that intact vesicles can be mobilized. For this, we co-deposited DiI-labeled liposomes with chitosan onto the 200-μm-wide gold electrode shown in Figure 7a (40 A/m^2 for 15 s). The red band observed in the fluorescence photomicrograph of Figure 7b shows co-deposition of the labeled liposomes. The patterned chip was then immersed in a 1 mL solution of 0.4 M Tris buffer (pH 6.0) and gently agitated. This treatment is known to solubilize chitosan. After 10 min of contact, the chip was removed from solution and examined. The fluorescence photomicrograph of Figure 7c shows that the red band is hardly visible, suggesting that the liposomes are mobilized by redissolving the electrodeposited chitosan.

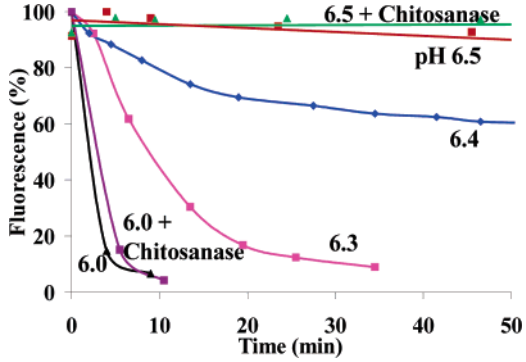

Figure 8. Sensitivity of vesicle mobilization to pH. DiI-labeled liposomes were co-deposited with chitosan and then incubated with gentle agitation in Tris-buffered solutions of varying pH. The addition of the chitosan-hydrolyzing enzyme chitosanase (0.1 U/mL) was not observed to enhance liposome mobilization under these conditions.

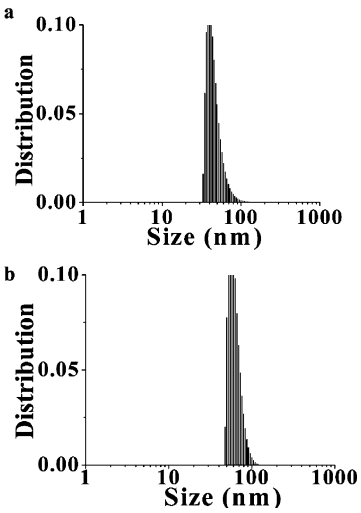

Figure 9. Dynamic light scattering evidence for the mobilization of intact liposomes from a deposit on an electrode surface. (a) Particle size distribution for initial liposomes. (b) Particle size distribution for liposomes recovered from a gold-coated wafer by acid treatment (to dissolve the chitosan matrix) and chitosanase treatment (to cleave the polymer and reduce the sample viscosity).

To study vesicle mobilization further, we co-deposited DiI-labeled liposomes with chitosan onto the gold electrodes (40 A/m^2 for 15 s) and then immersed these electrodes in Tris solutions buffered at different pH values. At various times, the chips were imaged using a fluorescence microscope, and the fluorescence of the deposit was quantified. Figure 8 shows little (if any) decrease in fluorescence for co-deposited films incubated at pH 6.5. Incubation at pH 6.0 resulted in the rapid solubilization of the deposited chitosan film with a corresponding decrease in fluorescence intensity. The addition of the chitosan-hydrolyzing enzyme chitosanase (0.1 U/mL) was not observed to enhance liposome mobilization. These observations suggest that liposomes co-deposited with chitosan are restrained within the film and resist dilution above pH 6.5; however, these liposomes can be

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mobilized by contacting the co-deposited film with slightly acidic solutions capable of dissolving the chitosan matrix.

Finally, we performed DLS measurements to demonstrate that acid-mobilized liposomes are intact. Analogous to the experiment described in Figure 4, we analyzed the initial liposome solution, and Figure 9a shows the size distribution of these liposomes. These liposomes were mixed with chitosan and electrodeposited (16 A/m^2 for 10 min) onto an unpatterned gold-coated wafer (8 $\times$ 8 mm^2) to deposit a sufficient number of liposomes for subsequent DLS analysis. After deposition, the chip was rinsed with distilled water and then immersed in 1 mL of acetate buffer (0.2 M, pH 5.0) to solubilize the chitosan matrix. Chitosanase (0.1 U/mL) was then added to cleave the polysaccharide, and the resulting solution was analyzed by DLS. Figure 9b shows the size distribution of the mobilized liposomes to be comparable to that of the original liposome solution. This result indicates that intact liposomes are mobilized by acid treatment of the electrodeposited matrix.

Conclusions

Vesicles are nanoscale structural units that biology employs to segregate and store quanta of chemical signals (e.g., neurotransmitters). Operations to restrain and mobilize vesicles by cytoskeletal association are integral to vesicle trafficking, and we mimic these operations using the aminopolysaccharide chitosan.[32] Specifically, we show that localized electrical signals can restrain vesicles through co-deposition with chitosan. These vesicles can be mobilized by the use of slightly acidic solutions (pH $<$ 6.5) that can resolubilize the chitosan film. Potentially, this work could extend the use of vesicles for microfluidic applications[33−41] by adding the capability for reversibly restraining vesicles at specific electrode addresses. This capability may enable multiple chemical reagents to be stored on-chip in segregated forms (i.e., in vesicles) for on-demand delivery to sites of sequential processing (e.g., to perform multistep assays). Potentially, this work could also provide new opportunities to interface electronic devices to neurobiological systems. Although electrodes are routinely used to report/alter ion-mediated intra-neuron signaling (i.e., action potentials), this work may provide a bridge between electrical and chemical signaling that allows electronic devices to interact with inter-neuron signaling that is mediated by chemicals (i.e., neurotransmitters).

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