Biosensors based on release of compounds upon disruption of lipid bilayers supported on porous microspheres

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The authors describe a biosensing concept based on the release of compounds, which are encapsulated within lipid-coated porous silica microspheres, by detergents and toxins that disrupt supported lipid bilayers (SLBs) on the microspheres. Suspension and microfluidic based methods have been developed to monitor the release of the encapsulated compounds in response to membrane disruption. The authors established that the SLBs on porous microspheres can endure experimental conditions necessary for their incorporation into packed microchannels while maintaining the bilayer integrity and functionality. Model compounds including a nonionic detergent (Triton X-100), a membrane active protein (α-hemolysin), and a membrane lytic antimicrobial peptide (melittin) were successfully utilized to interact with different formulations of SLBs on porous silica microspheres. The results demonstrate the stability of the SLBs on the microspheres for several weeks, and the feasibility of using this system to detect the release of fluorescent dyes as well as other molecular reporters. The latter were detected by their involvement in subsequent biospecific interactions that were detected by fluorescence. This study exemplifies proof of concept for developing new chemical and biochemical sensors and drug delivery systems based on the disruption of lipid membranes coating porous silica microspheres that encapsulate dyes or bioactive compounds. © *2008 American Institute of Physics.* [DOI: 10.1116/1.2918743]

I. INTRODUCTION

Supported lipid bilayers (SLBs) on microspheres serve as a biomimetic platform for several biotechnology applications such as biosensing, molecular interactions, and drug delivery.[1–14] Microsphere SLBs combine the advantages of maintaining a cell-like structure covered with lipid bilayers that mimic cell membranes while eliminating problems associated with using lipid vesicles.[4,5,7,12,15] Thus, the lipid bilayer allows for incorporation of integral membrane proteins and interactions with membrane active peptides while their support on microspheres introduces long term stability, rigidness, defined shapes and sizes, and ease of handling in comparison to lipid vesicles.[4,5,7,15,16] The lipid-coated microspheres can be useful for a variety of high throughput screening methods.

SLBs typically have a thickness of $\sim 5 \pm 1$ nm and were first stably formed on hydrophilic glass microspheres with a water interface (thicknesses of 1.2–2.2 nm) between the SLB and the microsphere surface.[15] SLBs on microspheres retain their fluidity,[7] which can be enhanced by modifying silica microspheres with a hydrophilic polymer to form a cushion for lipid bilayers.[17] Furthermore, direct patterning of SLBs on silica microspheres is achievable,[18] which can allow the presentation of biomolecules on microspheres in a controlled manner in restricted areas.

Besides solid silica microspheres, porous silica microspheres were used as supports for lipid bilayers.[4,7,8,19] Membrane proteins were successfully incorporated into SLBs on porous silica microspheres[4,7,8] while maintaining lateral mobility within the SLB.[7,8] Furthermore, recently, an artificial system of cellular membrane proteins from a human colonic adenocarcinoma cell line was reconstituted into SLB around porous silica microspheres.[19]

Silica microspheres, solid or porous, coated with lipid bilayers were used in assays of biomolecular interactions.[1–4,6–11,13,14] These studies detected biomolecular interactions by using various techniques, including measuring zeta potential changes,[9] evaluating dispersal of microspheres,[6,13] measuring ion conductance,[8] or by employing fluorescence measurements, whether or not in suspension, using a fluorimeter, a flow cytometer, or fluorescence

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microscopy,[1–3,8,10,14] or by packing solid microspheres coated with SLBs in microchannels with detection by using a fluorimeter.[11]

Microspheres with SLBs can be used in sensing schemes that employ disruption of the SLB with agents that can disrupt lipid bilayers or cell membranes. Detergents are an example of such agents that solubilize lipid membranes by forming detergent-lipid mixed micelles.[20,21] Membrane active proteins produced by microorganisms (e.g., α-hemolysin, streptolysin-O, and tetanus toxins),[22–27] antimicrobial peptides produced by microorganisms against other microbes (e.g., alamethicin, magainin, and gramicidin),[28–31] and components of venoms secreted by insects such as bees (melittin[30,32]) and black widow spiders (α-latrotoxin[33]) are a few of the biomolecules that are known to disrupt lipid membranes. Some membrane disrupting agents act by solubilizing the lipid bilayer, whereas others act through excessive formation of pores or channels, which leads to the complete disruption of the cell membranes or lipid bilayers. Such agents have been used mainly with lipid vesicles; on the other hand, we recently demonstrated the use of Triton X-100 to disrupt SLBs on microspheres in a detection format that employed a quencher and its superquenchable polymer.[14]

Porous microspheres coated with SLBs offer an advantage in biosensing applications and in drug delivery based on the accessibility of the internal pore volume of these microspheres from the surface. In fact, their pores were successfully loaded with fluorescent dyes and encapsulated with SLBs.[7,8] SLBs on porous microspheres can be impermeable to ions or molecules (e.g., dyes) and thus are capable of forming enclosed compartments that separate the internal and external chemical environments of the porous microspheres, in much the same way as observed in cells. This concept was confirmed when SLBs were used as barriers to external environment while encapsulating compounds, within hydrogel microspheres, which were released upon disruption of the lipid bilayer, thus allowing the interaction of the hydrogels and their load with the external environment.[34] Although porous microspheres have been used with SLBs to either incorporate functional membrane proteins or encapsulate fluorescent dyes stored within their pores, the utility of releasing compounds stored within the pores of the microspheres by disrupting the SLB has not been exploited.

The goal of this study is to evaluate the use of porous microspheres to release entrapped compounds by disrupting the microsphere SLB using various membrane disrupting agents. Our aims are to (1) characterize porous microspheres loaded with a fluorescent dye with SLB formed around them and evaluate their stability, (2) establish the stability of the SLB upon packing into a microchannel, (3) demonstrate the utility of lipid-coated porous microspheres in sensing by detecting the release of a fluorescent dye upon disruption of SLBs with membrane active agents in suspension assays and in a microchannel format, and (4) utilize SLB disruption to release nonfluorescent compounds that become involved in a subsequent interaction, which is detectable by fluorescence. The fourth aim was carried out in a microchannel containing two separated segments of microspheres. The first one had porous microspheres with SLBs to encapsulate biotin, and the second one was made of microspheres coated with streptavidin with bound fluorescein-conjugated biotin. The disruption of SLBs in the first segment released biotin, which, in turn, interacted with streptavidin in the second downstream segment. The fluorescence detection was based on the dequenching of ostrich-quenched fluorescein biotin[35,36] caused by the released biotin from the first segment. The ostrich quenching of fluorescein conjugated to biotin results from the interaction of fluorescein with a *cis* binding pocket on streptavidin.[35] The addition of biotin blocks ostrich quenching, which leads to the increased emission intensity of fluorescein.

In the present study, we showed that SLBs on porous microspheres can stably encapsulate molecules for several weeks. We also demonstrated the proof of concept for developing sensors and drug delivery systems based on the disruption of lipid membranes coating porous silica microspheres loaded with fluorescent or nonfluorescent compounds. In this proof-of-concept study, we successfully used several model compounds for lipid bilayer disruption: a nonionic detergent (Triton X-100), a membrane active protein (α-hemolysin), and a membrane lytic antimicrobial peptide (melittin).

II. MATERIALS AND METHODS

A. Materials

Egg phosphatidyl choline (EPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG) were purchased from Avanti Polar Lipids (Albaster, AL). Porous silica microspheres (50 Å nominal pore size, 5 μm diameter) were from Macherey-Nagel (Easton, PA). Nonporous silica microspheres (5 and 20 μm diameter) were purchased in dry form from Duke Scientific (Palo Alto, CA). Streptavidin-coated polystyrene microspheres with 20 μm diameter were purchased from Spherotech, Lake Forest, IL. Fluorescein sodium salt, Triton X-100, and melittin were purchased from Sigma. α-hemolysin/α-toxin was purchased from Calbiochem (San Diego, CA). NH_4OH, H_2O_2, and HCl were purchased from VWR (West Chester, PA). De-ionized ultrapure water (Barnstead International, Dubuque, IA) was used throughout all experiments.

B. Preparation of fluorescein- or biotin-loaded microspheres

Silica microspheres were cleaned and treated with a mixture of 4% NH_4OH and 4% H_2O_2 at 80 °C for 10 min. The microspheres were then rinsed in distilled water once and treated with a mixture of 4% HCl and 4% H_2O_2 at 80 °C for 10 min. Microspheres were rinsed in distilled water five times and suspended in distilled water. Portions of microspheres (5 mg) were suspended in microcentrifuge tubes con-

taining 200 μl of 10 mM fluorescein or 25 mM biotin in tris buffer (100 mM tris, 150 mM NaCl, *p*H 7.4) for a minimum of three days.

C. Formation of supported lipid bilayers on microspheres

Fluorescein- or biotin-loaded microspheres were separated from fluorescein or biotin solution by centrifugation and coated with a single lipid bilayer as described elsewhere.[7,11,15] Briefly, the lipid (or mixture of lipids) (1 mM) in chloroform was dried under a stream of nitrogen followed by vacuum for half an hour. Unilamellar vesicles[15] were prepared by hydrating the dry lipids with tris buffer followed by sonication (Aquasonic, model 500, VWR) for 20 min. Ice was added to the sonicator bath if necessary to maintain ambient temperature. Lipid bilayers were formed around the microspheres by a vigorous shaking of the microspheres with a suspension of unilamellar vesicles using a Vortex mixer for 5 min followed by a 25 min incubation without shaking. Excess lipids and fluorescein dye were removed by rinsing the lipid-coated microspheres in tris buffer at least 15 times. The microspheres were suspended in tris buffer at a concentration of 5 mg/ml.

D. Scanning electron microscopy and confocal scanning laser microscopy

The porous silica microspheres were characterized by scanning electron microscopy (SEM) (Hitachi S5200, 1 kV). Lipid-coated fluorescein-containing microspheres were imaged by using a confocal scanning laser microscope (Zeiss LSM510) equipped with an argon-ion laser.

E. Fluorimetry

Fluorescence measurements were performed by excitation at 488 nm and collection of emission at 520 nm using a Fluorolog-3 SPEX fluorimeter (Instruments S.A., Edison, NJ).

F. Disruption of SLBs in suspension assays

A tube containing 200 μL of lipid-coated microspheres that encapsulated fluorescein was divided into two tubes. The two aliquots were centrifuged and their supernatants were mixed, in separate centrifuge tubes, with equal volumes of either tris buffer or a membrane disrupting reagent. The fluorescence intensity of each mixture was immediately (time =0) recorded. Each mixture was mixed with its respective microsphere sample in centrifuge tubes and incubated at 25 °C in the dark. After 30 min, the samples were centrifuged and the fluorescence intensity of the supernatants was measured. Microspheres were resuspended in their respective supernatants. This process was repeated again and fluorescence intensities were measured at 90 and 150 min. The disrupting agents tested were (1) Triton X-100 at a final concentration of 5% (w/v), (2) α-hemolysin, where three different toxin concentrations: 15, 45, and 250 μg/ml were used at 37 and 25 °C with microspheres coated with EPC, or (3) melittin at a final concentration of 220 μM with microspheres coated with either 100% EPC, 10:90 (molar ratio) mixture of DMPG and EPC, or an 80:20 mixture of DMPG and DMPC. All melittin experiments were conducted at 25 °C.

G. Detection of SLB disruption by α-hemolysin using flow cytometry

Fluorescein is a *p*H-sensitive dye whose fluorescence is significantly reduced below *p*H 7.0.[37] We employed this property by encapsulating fluorescein by SLBs in an acidic environment in the porous microspheres while suspending the microspheres in a basic environment. The addition of α-hemolysin will create pores within the SLB, which allows the movement of protons from the internal acidic environment of the microspheres to the external basic environment, thus raising the *p*H within the pores where the fluorescein is stored and causing an increase in fluorescence. For that reason, 12×10^6 porous microspheres were suspended in 1 mM fluorescein in the flow cytometry sheath buffer, in which the *p*H was adjusted to 2.6 in a total volume of 600 μl, and incubated overnight with shaking at room temperature. The microspheres were sedimented by centrifugation and then incubated with 1 mM EPC in 400 μl sheath buffer (*p*H 2.6) to form a SLB as described above. Excess lipids and fluorescein dye were removed by rinsing the lipid-coated microspheres in sheath buffer four times, in which the *p*H was adjusted to 11.0. The microspheres were then resuspended in 400 μl of sheath buffer that has a *p*H of 11.0. An α-hemolysin stock solution of 200 μg/ml was prepared in sheath buffer (*p*H 7.4) and kept on ice until used. α-hemolysin was added to the prepared microspheres at concentrations of 0, 5, 15, and 45 μg/ml and was incubated immediately at 37 °C with stirring. Fluorescence readings were measured every 2 min while incubated at 37 °C by using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA) with excitation at 488 nm. Fluorescence signals were acquired on the FL-1 channel (525 nm) by using log amplification and were analyzed with the CellQuest™ software.

H. Preparation of microfluidic channels

Polydimethylsiloxane (PDMS) microchannels were constructed by using soft lithographic techniques adapted from the literature.[38] The microfluidic channels were fabricated with weirs to hold the microspheres in place as described elsewhere.[11] The dimensions of the microchannel were 2 cm in length, 250 μm in width, and 60–70 μm in height. In order to trap the microspheres, the depth of the channel was limited to 12–15 μm near the outlet. The prepared PDMS channel was irreversibly sealed onto a glass slide after exposing both to an Ar plasma.[38]

I. Release of fluorescein upon disruption of SLBs in a microchannel format

To create a packed microcolumn of lipid-coated microspheres containing fluorescein, 2 μl of silica microsphere (diameter=20 μm) suspension (0.05 mg/μl) were injected into a microfluidic channel by applying vacuum at the outlet port of the microchannel, followed by the injection of 10 μl of fluorescein-entrapped microsphere (diameter=5 μm) suspension (0.05 mg/μl). The microsphere-packed microchannel was mounted onto a vertical translational stage located in the sample holder space of the fluorimeter.[11] Just below the microsphere segment, the microchannel was irradiated with an argon ion laser (λ=488 nm excitation, 8–10 mW). The inlet of the column was connected to a buffer reservoir, while the outlet was connected to a vacuum source. As the fluorescence intensity at 520 nm was monitored, several microliters of tris buffer were passed through the microchannel before the injection of Triton X-100. To avoid photobleaching, we irradiated the microchannel right beneath the microsphere segment, where the disruption of bilayers was detected by monitoring the diffusion of fluorescein from the porous microspheres into the buffer stream at the irradiated area. After about half an hour, 10 μl of 10% (w/v) Triton X-100 was injected into the column through the inlet silicon tubing by using a Hamilton syringe.

J. Detection of membrane interactions by downstream biomolecular interactions of a compound released upon SLB disruption

A microchannel was packed with a segment (~1 mm) of streptavidin-coated microspheres (20 μm diameter) to which a nonsaturating amount of fluorescein biotin was bound.[39] This was followed by a segment (~1 mm) of blank silica microspheres (20 μm diameter) and a segment (~2 mm) of lipid-coated porous microspheres containing biotin. The blank silica microspheres served as the spacer to prevent mixing of fluorescein biotin microspheres with biotin encapsulated microspheres. Biotin-loaded microspheres were encapsulated with an 80:20 (molar ratio) mixture of DMPG and DMPC. A 10 μl aliquot of either 10% (w/v) Triton X-100 or 220 μM melittin was injected to the column. The fluorescein biotin microsphere segment was irradiated at 488 nm and the increase in its fluorescence emission intensity was measured at 520 nm every 10 min.

III. RESULTS AND DISCUSSIONS

A. Characterization of microspheres with SEM and confocal microscopy

SEM of the porous microspheres used in this study [Fig. 1(a)] shows that they are spherical, with ~90% of the microspheres having an average diameter of ~5 μm (~10% of the microspheres had a smaller diameter of ~1–2 μm). Figures 1(b) and 1(c) show high resolution SEM images of the surface of the microspheres. The manufacturer specifies a nominal pore diameter of 50 Å; however, from the SEM image, it is clear that the pores are not regular in size and also that the microspheres contain a fraction of pores that is bigger than 50 Å. Silica microspheres are negatively charged at a pH of 7.5.[40] Fluorescein is also negatively charged at this pH; nevertheless, incubating the porous microspheres in highly concentrated solutions for one day was sufficient to load the microspheres with detectable amounts of fluorescein. For our experiments, we incubated the microspheres in fluorescein for three days. After coating the fluorescein-loaded microspheres with lipid bilayers, the microspheres were rinsed with tris buffer 15 times. Rinsing the microspheres even three times results in the formation of one SLB rather than multilayers,[8] but to remove excess fluorescein, extra rinsing was needed. A confocal fluorescence image of the lipid-coated microspheres packed into a microcolumn [Fig. 1(d)] demonstrates that the dye is encapsulated inside the microspheres.

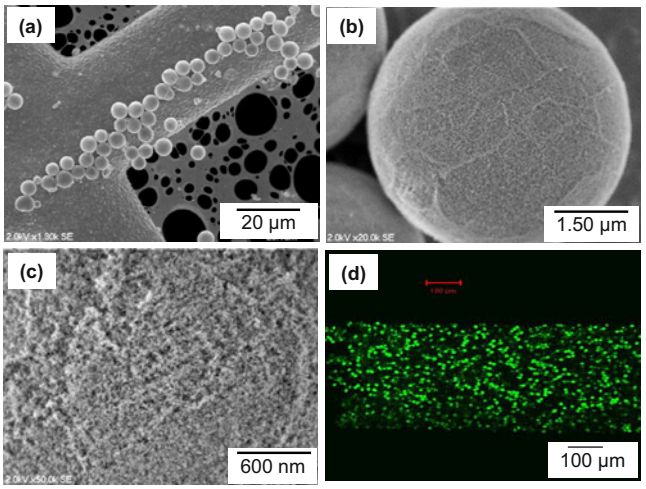

FIG. 1. Images of porous microspheres. (a) SEM image of porous silica microspheres (average diameter=5 μm). (b) High resolution SEM image of a porous silica microsphere surface. (c) Higher resolution image of a porous silica microsphere showing its porous surface. (d) Confocal fluorescence image of microspheres containing fluorescein encapsulated in lipid bilayer membranes packed in a microcolumn.

B. Long term stability of a lipid bilayer supported on porous microspheres

The stability of SLBs on fluorescein encapsulated microspheres was evaluated by monitoring the leakage of fluorescein into solution (Fig. 2). This was done by collecting the supernatant after sedimenting the microspheres, as explained under Sec. II. Monitoring was done for a period of one month for microspheres that were stored at 25 and 4 °C. Since the temperature can influence the fluorescence intensity,[41] the fluorescence of the supernatant was always measured at room temperature. At the end of a one month period, SLBs on microspheres were disrupted with 10% (w/v) Triton X-100 in tris buffer and incubated for 30 min. We assumed that this addition of Triton X-100 will completely disrupt the SLB and cause 100% leakage as we demonstrated an ~70-fold increase in fluorescence intensity of the supernatant upon disruption of the SLBs with Triton X-100 [Fig. 4]. Controls included lipid bilayer coated blank

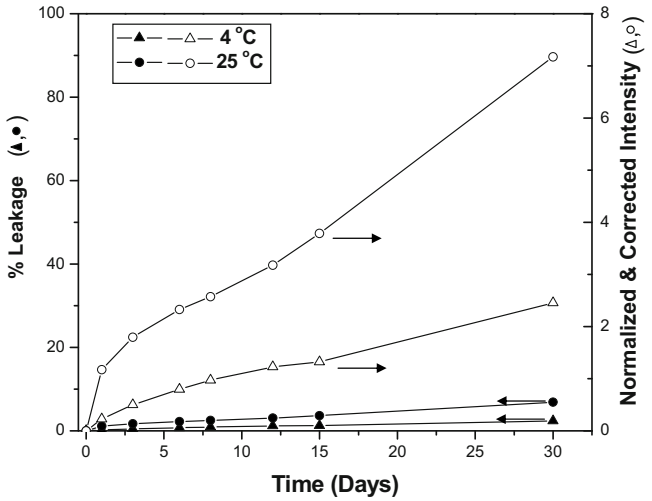

FIG. 2. Stability of lipid bilayers on porous microspheres. Normalized and corrected intensity of supernatant fluorescence intensity (open symbols, *y* axis to the right) and percent leakage (closed symbols, *y* axis to the left) are plotted against time. See text for details on calculating the normalized and corrected intensity and percent leakage. This figure is representative of three replicates.

porous microspheres and 1 μM fluorescein solutions kept at 25 and 4 °C. All the intensities were normalized to the intensity at day 1 and corrected for any possible fluctuations in efficiency of fluorescence excitation or emission detection during the experimental period by using fluorescein solutions (1 μM, 4 and 25 °C) as controls according to the following:

$$I = \left(\frac{I_i - I_0}{I_0}\right) - \left(\frac{I_{\mathrm{flc}} - I_{\mathrm{flc},0}}{I_{\mathrm{flc},0}}\right), \quad (1)$$

where I is the normalized and corrected intensity of the supernatant for each day, I_i is the fluorescence intensity measured for the supernatant each day, I_0 is the fluorescence intensity of the supernatant on day 1, I_{flc} and $I_{\mathrm{flc},0}$ are the intensities of the control fluorescein solutions on each day and on day 1, respectively. The observed fluctuation in intensity of the controls was negligible. The leakage of the dye during the one month period is illustrated in Fig. 2, as represented by the traces with open symbols. The total fluorescence intensity increase at the end of the one month period was ~7.2-fold at 25 °C and ~2.5-fold at 4 °C relative to their corresponding initial readings.

The percent leakage of the dye into solution, relative to that caused by adding Triton X-100, was calculated according to the following:

$$\% \quad \mathrm{Leakage} = \left(\frac{I_i - I_0}{I_{\mathrm{Trt}} - I_0}\right) 100, \quad (2)$$

where I_{Trt} is the total release of the dye due to the disruption by Triton X-100. The traces with closed symbols in Fig. 2 represent the percentage of leakage of the dye. The percentage of leakage of fluorescein into solution after 30 days for lipid-coated microspheres kept at 25 °C was ~6.9%, while that of microspheres kept at 4 °C was ~2.4%. It is known that lipid bilayer membranes are more stable at lower temperatures.[42] The greater amount of fluorescein leakage through the SLB observed at 25 °C may be due to the increased lateral diffusion of lipid molecules as well as the increased rate of diffusion of fluorescein. At both temperatures, the leakage is very low, and for the purpose of the types of assays described here, this leakage is considered negligible. In this study, we formed the SLB by using EPC, which is a mixture of saturated and unsaturated lipids with different molecular sizes and phase transition temperatures.[43] The phase transition temperature of EPC is below 0 °C, and at both 25 and 4 °C, the membrane is in a fluid phase. The leakage might be further minimized by using a suitable lipid composition that has a phase transition temperature higher than that of EPC.[42]

Our data suggest that the lipid membranes on microspheres are relatively stable for at least one month. In comparison, lipid vesicles cannot be stored for more than one week without a substantial loss of encapsulated compounds, and the preparation of fresh vesicle samples is often required for long term studies. Thus, the use of robust supported lipid bilayers on porous silica microspheres can have advantages over the use of lipid vesicles in applications requiring maintenance of encapsulation properties over a long period of time as they are easier to handle and are more uniform in size than lipid vesicles.

C. Detecting SLB disruption in suspension assays

Previous suspension studies on disruption of lipid bilayers were performed by using small unilamellar vesicles.[26,44] In those experiments, the disruption of the lipid bilayer membranes was detected by monitoring the unquenching of a highly concentrated encapsulated dye.[26] In the present work, disruption is monitored via release of fluorescein dye from porous microspheres, by measuring the increase in fluorescence intensity of the supernatant at 520 nm. As depicted in Fig. 3(a), microspheres were first suspended in tris buffer containing the disruptor. Microspheres can be easily sedimented by centrifugation; thus, a portion of the supernatant can be taken for measurement. The measured portion was added back to the microsphere sample for semicontinuous monitoring. The scattering effect that is present in vesicle based studies can be minimized by measuring the fluorescence of a microsphere-free supernatant.[45,46]

1. Detecting the disruption of SLBs by Triton X-100 in suspension assays

Triton X-100 can disrupt lipid membranes within milliseconds.[20] As depicted in the schematic in Fig. 3(b), the encapsulated dye will be released when the SLB is solubilized by Triton X-100. An evaluation of the dye release upon disrupting the SLB by adding Triton X-100 was carried out by plotting the fluorescence intensity of the released dye in the supernatant, normalized to the initial fluorescence intensity reading, versus time (Fig. 4). The disruption of SLBs encapsulating fluorescein in microspheres by Triton X-100 caused an increase of more than 70-fold in fluorescence intensity of the supernatant when examined after 30 min of

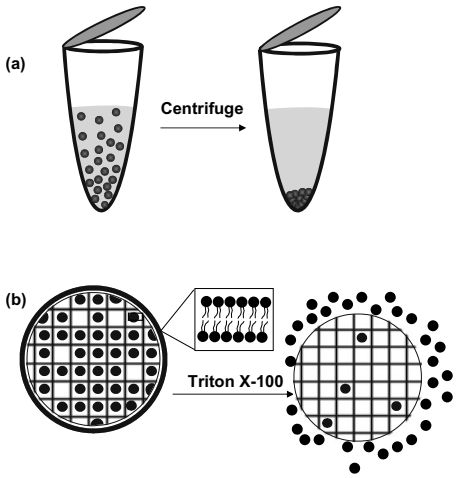

FIG. 3. Suspension assay. (a) Schematic representation of suspension study. Initially, the microspheres are suspended with the disruptor. Solution will be separated from microspheres by centrifugation and analyzed for fluorescein released by the microspheres. (b) Schematic representation of total disruption of SLBs on porous silica microspheres with fluorescein dye inside. Triton X-100 will solubilize the entire bilayer while membrane active peptides and proteins will form channels or pores through the membrane.

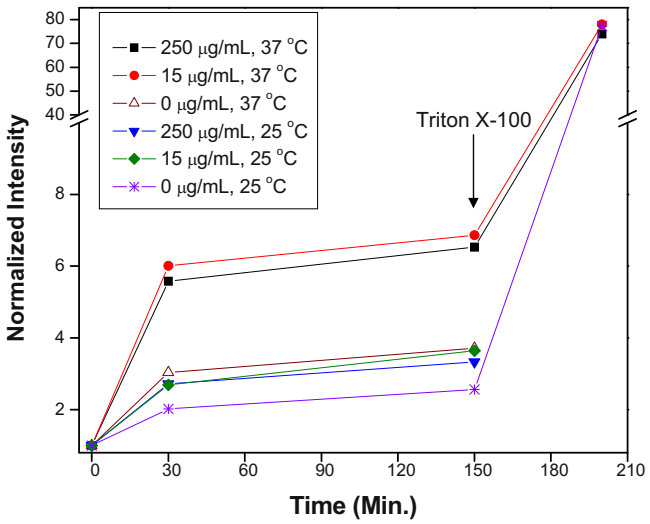

FIG. 5. Detecting the disruption of SLBs by α-hemolysin in a suspension assay. Normalized fluorescence intensity (at 520 nm) vs time for suspension assays of EPC-coated, fluorescein-filled porous microspheres with different concentrations of α-hemolysin added. 10% (w/v) Triton X-100 was added after 150 min. This figure is representative of three replicates.

incubation. There was no significant change in the fluorescence intensity of the supernatant after 30 min, suggesting relatively rapid equilibration of the release of fluorescein. The leakage in the absence of Triton X-100 is shown in the trace with filled rectangles in Fig. 4. The trace with filled circles represents a control sample of lipid-coated porous microspheres without fluorescein. These results suggest that Triton X-100 is an efficient disruptor of lipid membranes supported on porous silica microspheres and that the release of the dye can be viewed as an effective measure of membrane disruption.

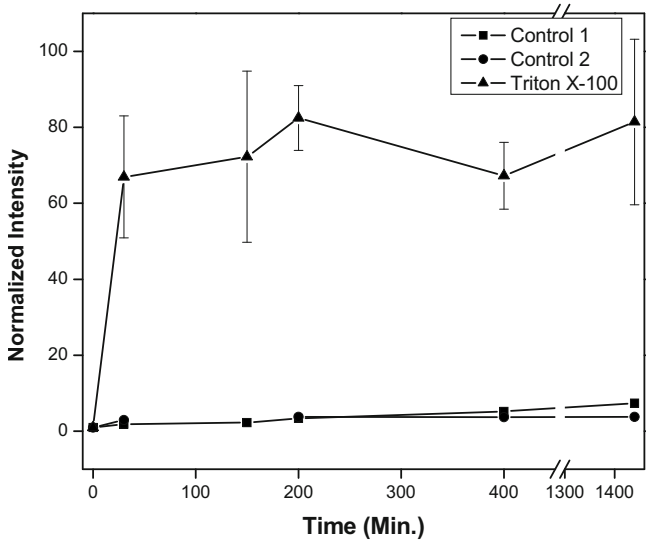

FIG. 4. Detecting disruption of SLBs by Triton X-100 in a suspension assay. The normalized fluorescence intensity at 520 nm of supernatants was plotted against time for suspension assays of EPC bilayer disruption by Triton X-100 (triangles). The fluorescence intensity is normalized to the original intensity. Squares represent the control with fluorescein encapsulated microspheres with no Triton X-100 added. Circles represent the control with empty porous microspheres coated with lipid bilayer, and Triton X-100 is added. This figure is representative of three replicates.

2. Detecting the interaction of α-hemolysin with SLBs in suspension assays

We examined the interaction of membrane active biomolecules, such as α-hemolysin and melittin, with the SLBs in suspension assays. α-hemolysin is a membrane active protein that is secreted by *Staphylococcus aureus* and has a molecular weight of ~33 kDa. α-hemolysin is also referred to as α-toxin, although the latter is the precursor of α-hemolysin with a propeptide sequence of 26 amino acids, and its molecular weight is ~36 kDa.[47] α-hemolysin forms heptameric pores ~1–2 nm in diameter in phospholipid bilayers.[22] The poration of membranes by α-hemolysin does not depend on the charge of the lipids, and the toxin is most reactive at 37 °C.[22,48] Suspension assays were performed (as described above for Triton X-100) at 25 and 37 °C for three different toxin concentrations: 15, 45, and 250 μg/ml. The release of fluorescein dye upon exposure of the microspheres to α-hemolysin was monitored over time and is presented in Fig. 5. The increase in fluorescence intensity at 150 min in comparison to 0 min was ~6.5-fold at 37 °C and ~3-fold at 25 °C for all three concentrations (data for 45 μg/ml of toxin are not shown in the figure for clarity). The release of similar quantities of the dye for all three concentrations indicated the presence of an adequate amount of α-hemolysin in the medium. The data confirmed the enhanced activity of the toxin at 37 °C in comparison to 25 °C. The addition of Triton X-100 after 150 min caused a total disruption of bilayers, resulting in an increased fluorescence intensity of 50-fold. This indicated that a large amount of dye remained in

the porous microspheres after the exposure of the SLBs to α-hemolysin.

The interaction of α-hemolysin with the SLB in comparison to Triton X-100 resulted in a smaller amount and rate of dye release. This is not unexpected, considering the toxin's mechanism of membrane interaction, as the release is affected by the size and the amount of pores formed by the toxin on the lipid membranes. This observation is consistent with reports that α-hemolysin will not sufficiently disrupt the lipid bilayers of vesicles to release all the encapsulated dye; for example, only 27% of the dye encapsulated in EPC vesicles was released upon incubation with α-hemolysin.[26] We investigated several different lipid formulations, which contain different quantities of cholesterol and phosphatidyl ethanolamine along with EPC, in an effort to enhance the disruption by α-hemolysin (data not presented); none of the lipid mixtures enhanced the degree of dye leakage beyond that observed with EPC alone. The toxin concentrations we used in this study to cause a detectable interaction were higher than those used in many published works,[49–51] which may be due to the stability of the bilayers of the chosen lipids on solid supports in comparison to lipid vesicles or the assay format.

3. Detecting the interaction of α-hemolysin with SLBs using flow cytometry

To evaluate whether using a different assay format would allow us to detect the activity of lower α-hemolysin concentrations, we performed a flow cytometry assay, in which the detection was based on encapsulating a *p*H-sensitive dye. Fluorescein is a *p*H-sensitive dye, whose fluorescence is significantly reduced below a *p*H of 7.0.[37] This property was employed in our sensing system, in which fluorescein was encapsulated by SLBs in an acidic environment in the porous microspheres while suspending the microspheres in a basic environment. We expect that the addition of α-hemolysin will create pores within the SLB, which will allow the movement of protons from the internal acidic environment of the microspheres to the external basic environment, thus raising the *p*H within the pores where the fluorescein is stored and causing an increase in fluorescence. Flow cytometry is a very sensitive method that allows the simultaneous measurement of multiple fluorescence signals as a result of the illumination of single cells or microscopic particles in suspension as they flow rapidly through a laser beam. Microspheres have been used in a variety of flow cytometric assays for biosensing and detecting molecular interactions.[7,52–54] We used flow cytometry to detect interactions of α-hemolysin with the SLB at 37 °C; in this case, directly measuring the fluorescence of the microspheres, rather than examining the fluorescence of the supernatant as we did in the suspension assays.

We determined the fluorescence every 2 min and normalized the reading to the fluorescence reading before adding α-hemolysin (0 min reading). Figure 6 shows that without adding α-hemolysin, there was a slight increase in fluorescence over time. This may be due to the leakage of protons

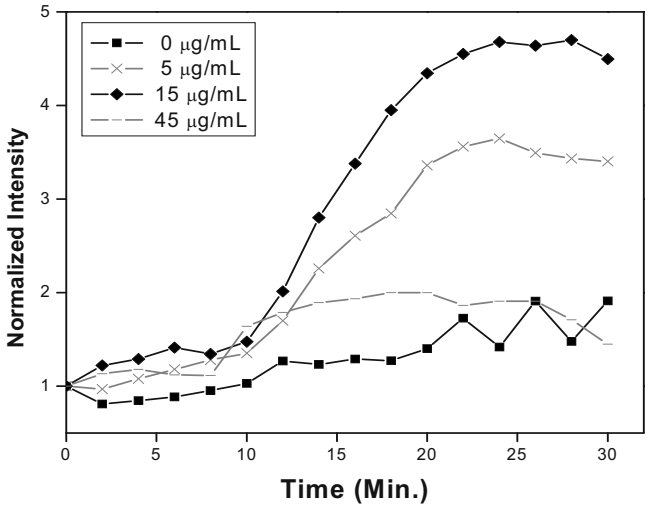

FIG. 6. Detecting the disruption of SLBs by α-hemolysin by flow cytometry. Normalized fluorescence intensity (at 525 nm) vs time for suspension assays of porous microspheres filled with *p*H-sensitive fluorescein and those coated with EPC with different concentrations of α-hemolysin added and incubated at 37 °C. The *p*H outside the microspheres was 11.0, whereas inside it was 2.6. This figure is representative of three replicates.

from the SLB-encapsulated microspheres. However, upon addition of α-hemolysin, at concentrations of 5 and 15 μg/ml, there was an increased fluorescence caused by permeation of the SLBs. This increase in fluorescence for both concentrations was initially slow at the beginning, then increased after 10 min and reached a plateau at around 24 min. The α-hemolysin concentration of 45 μg/ml caused only a slight increase in fluorescence. We also tested other concentrations of α-hemolysin, including 2.5, 10, 25, and 30 μg/ml. All these concentrations gave a curve similar to that of 5 μg/ml α-hemolysin (data not shown). Furthermore, upon monitoring all the seven concentrations of the α-hemolysin tested for a longer time, the fluorescence gradually declined to 1 after 76 min. Our findings indicated that α-hemolysin permeated the SLB and allowed proton movement, which was detectable by the increase in the fluorescence of the *p*H-sensitive dye fluorescein. However, with time, a plateau was reached, which was followed by a decline in fluorescence that may be caused by two factors: one is the equilibration of protons and hydroxyl ions across the SLB, and the second is the possible leakage of fluorescein from the particles after excessive pore formation by α-hemolysin. The higher concentration of α-hemolysin (45 μg/ml) seems to have caused excessive poration of the SLB. This may either allow a faster equilibration of protons and hydroxyl ions across the SLB or, alternatively, cause a faster leakage of fluorescein, and thus, no significant change in fluorescence was detected. This assay format enabled us to detect the action of α-hemolysin at concentration as low as 2.5 μg/ml, although the optimal response was obtained at a concentration of 15 μg/ml.

4. Detecting the interaction of melittin with SLBs in suspension assays

We also examined the disruption of SLBs in suspension assay by using the membrane active peptide, melittin. Melit-

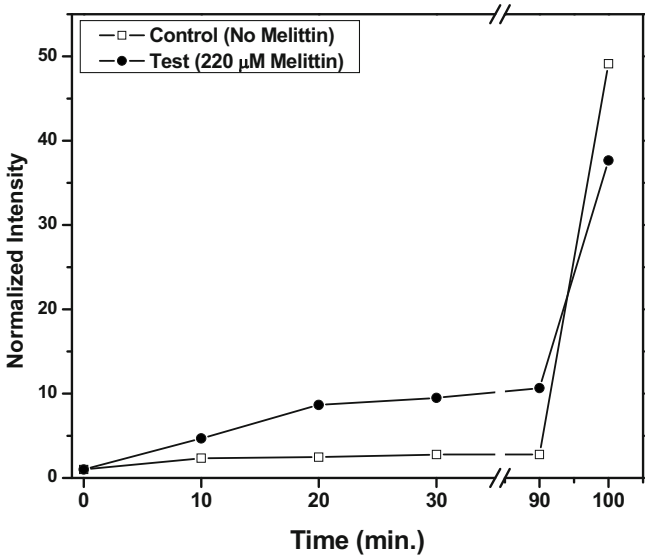

FIG. 7. Detecting the disruption of SLBs consisting of (A) EPC or EPC/DMPG or (B) DMPG/DMPC by using melittin in a suspension assay. Normalized fluorescence intensity (at 520 nm) vs time for suspension studies with 220 μM melittin. Microspheres were coated either with EPC, 10:90 (molar ratio) mixture of DMPG and EPC, or 80:20 (molar ratio) mixture of DMPG and DMPC. 10% (w/v) Triton was added after 90 min. The open symbols represent the controls in the absence of melittin. This figure is representative of three replicates.

tin, the principal toxic component in bee venom, is an antimicrobial peptide with 26 amino acid residues that has a strong interaction with negatively charged lipids. Melittin tends to form pores in lipid membranes with zwitterionic lipids; however, it can disrupt membranes of negatively charged lipids in a "detergentlike" action,[55–57] although a higher concentration of melittin is required for the latter effect.[57] On the other hand, when anionic lipids are added to zwitterionic lipids in vesicles, this confers resistance to melittin action.[58,59]

We examined the interaction of melittin with SLBs consisting of one of the following: 100% EPC, a 90:10 mixture (molar ratio) of EPC and DMPG (a mixture of a zwitterionic lipid with a negatively charged lipid), or 80:20 mixture (molar ratio) of DMPG and DMPC (a mixture of a negatively charged lipid with a length-matched zwitterionic lipid). To evaluate the interaction with these different SLB compositions, we performed suspension assays by exposing to aqueous solutions of melittin at room temperature. We found that the results for the three lipid mixtures tested were comparable; hence, we are presenting here only the results for the 80:20 (molar ratio) mixture of DMPG and DMPC (Fig. 7). The addition of melittin led to a gradual release of the encapsulated fluorescein (Fig. 7). The release of fluorescein from porous microspheres coated with the different lipid mixtures was noticed as early as 10 min after incubating with melittin. In the absence of melittin, the leakage was low (Fig. 7), which is important as these microspheres can be used in studies for a longer period of time without losing their sensitivity.

From these observations, we conclude that melittin, whether in the presence or in the absence of DMPG in bilayers, resulted in the release of an encapsulated dye from porous microspheres through enhanced toxin-membrane interactions. We could not evaluate if melittin would interact in a detergentlike fashion with a SLB composed of only anionic phospholipids because these lipids are negatively charged, and so are our microspheres, which may cause instability of the SLBs.

Although the release of fluorescein caused by melittin is not high as in the case of Triton X-100, it is notable in comparison to the release observed with α-hemolysin. This experiment demonstrates the possibility of forming bilayers composed mainly of negatively charged lipids on negatively charged porous silica microspheres. The ability to form lipid bilayers, consisting of mixtures of negative and zwitterionic lipids on silica surfaces, was reported previously[60,61] and it is thought that negative lipids will partially segregate to the outer leaflet of such membranes.[61] Although we used a higher molar fraction of DMPG than that of DMPC, there is a possibility that the membranes formed had DMPG-rich outer leaflets. Detailed spectroscopic studies will be necessary to characterize the likely complex structure of such lipid bilayers on porous silica microspheres. The melittin experiments suggest the possibility of developing silica microsphere-based membrane-toxin interaction studies for other membrane active cationic peptides.

Although the reports in literature showed that the disruption of lipid membranes either by membrane active peptides or proteins can release a large amount of encapsulated compounds from unilamellar vesicles,[22,27,32] we did not observe this with lipid-coated porous silica microspheres. One can argue the possibility of having multilayers of lipid bilayers around the microspheres; however, we previously demonstrated that this is not the case.[8] On the other hand, although the effect of the silica support on the lytic activity of membrane active compounds is not well understood, some studies suggest that, by increasing the interfacial distance between lipid bilayers and the support, the disruption can be enhanced.[24,62]

D. Detecting SLB disruption in microfluidic format

Having analyzed the detergent's and toxins' capabilities of interacting with membranes supported on microspheres in suspension, we next explored the possibilities of developing microfluidic chemical and biochemical sensors to detect membrane-detergent and membrane-toxin interactions. These microfluidic studies were based on the detection of the release of a fluorescent dye or a nonfluorescent compound from porous microspheres packed into a microcolumn inside a microfluidic channel. First, we wanted to demonstrate that we could pack lipid-coated porous microspheres in a microcolumn without causing membrane disruption during the packing process. Figure 8(a) shows the optical micrograph of a SLB-microsphere packed microcolumn. The packed segment was ~ 2 mm long. The monitoring of the released fluorescein from the microspheres was performed at a point just

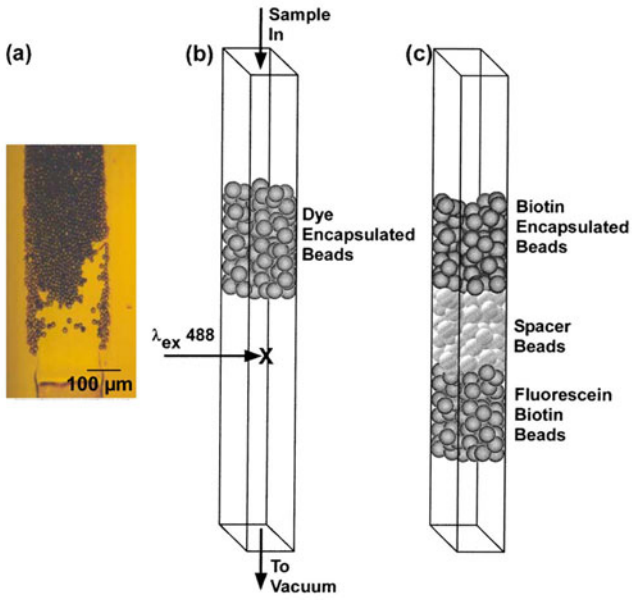

FIG. 8. Microcolumn configurations for membrane interaction analyses. (a) Optical micrograph of a microsphere packed microcolumn. Microspheres are retained by having a weirlike structure at the bottom of the microcolumn. (b) Schematic of the microcolumn showing the detection point for the phospholipid bilayer disruption study with Triton X-100. The detection point (marked with *X*) is irradiated with a 488 nm Ar-ion laser beam. (c) Schematic of the microfluidic biosensor based on bilayer disruption. A microcolumn is packed with a 1 mm long fluorescein-biotin-coated streptavidin microsphere segment at the bottom with a stacked 1 mm long spacer microsphere segment and, finally, a 2 mm long biotin encapsulated microsphere segment.

below the microsphere segment, as indicated in Fig. 8(b). Since we monitor a region that is originally nonfluorescent, the alignment of the microcolumn with the excitation source and the detector is challenging. To overcome this, we first aligned the fluorescent microsphere segment with the beam from an argon ion laser and the detector, then vertically moved the column to a suitable region while monitoring the fluorescent signal from the microcolumn, as shown in Fig. 9(a). The detection region should be downstream from the microsphere segment, where the signal does not interfere with the one that arises from adjacent fluorescent microspheres. The microcolumn was irradiated at 488 nm and fluorescence intensity was monitored at 520 nm.

1. Stability of SLBs on microspheres packed into a microchannel

The packing process of the microspheres in a microcolumn or the close contacts of the packed microspheres can cause disruption or instability of the SLBs on these microspheres. We evaluated the latter possibility by examining the stability of SLBs on porous microspheres, containing fluorescein dye, by monitoring the leakage of the dye from microspheres. Figure 9(b) shows the fluorescence intensity of the detection region, at flow and no flow conditions. A tris buffer was flowed through the column ($\sim$1.2 μl/min) for $\sim$15 min and then stopped for another 15 min. Had the dye

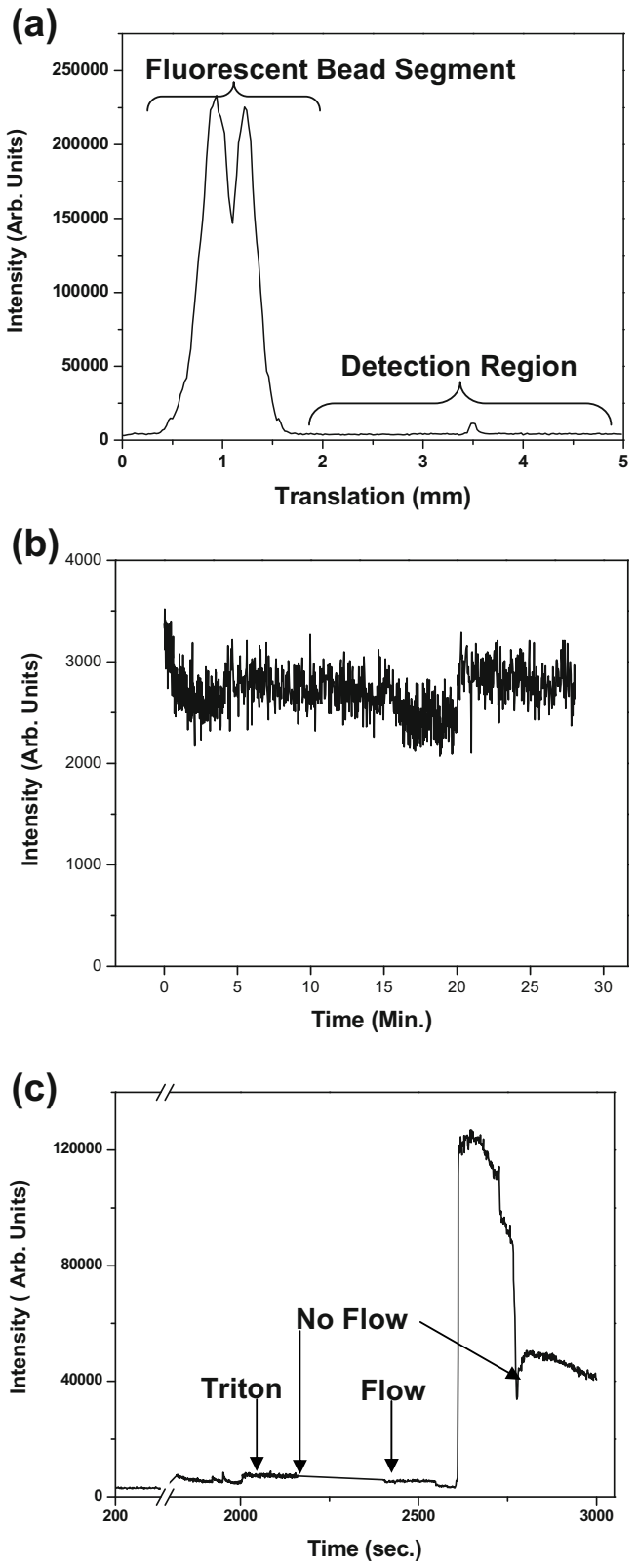

FIG. 9. Stability and Triton X-100 disruption of SLBs in microfluidic studies. (a) Alignment of the microsphere packed microcolumn for fluorimetry experiments. (b) Microfluidic stability study. The very low intensity indicates the stability of lipid-coated microspheres when packed in the microcolumn. (c) Microfluidic disruption upon introduction of 10% (w/v) Triton X-100. This figure is representative of three replicates.

leaked out to a significant extent, it would have been transported into the buffer stream and increased the fluorescence intensity at the detection region. However, no significant leakage of the encapsulated dye occurred from the lipid membrane-coated porous microspheres, which indicated that the SLBs were stable enough to withstand packing into a microcolumn. Thus, we were confident that packing lipid-coated porous microspheres into microcolumns does not disrupt the SLBs to a significant extent.

2. Detection of membrane interaction by release of a fluorescent dye

We evaluated this microfluidic sensor in detecting the release of encapsulated fluorescein upon disruption of the SLBs by using Triton X-100. After injecting 10 μl of 10% (w/v) Triton X-100 into the fluorescein encapsulated microsphere containing microcolumn, microspheres were incubated with Triton X-100 at no flow condition for about 5 min. The flow was then started to move the released fluorescein dye to the detection point. As Fig. 9(c) shows, a 25-fold increase in fluorescence intensity was observed. The following decrease in fluorescence intensity was due to the dilution of the released dye in the transport buffer. Following that and in the absence of flow, the intensity was nearly constant. This suggested that the dye was relatively immobile in the absence of buffer flow. The increase in fluorescence intensity due to Triton X-100 disruption of the SLBs obtained here was less than that observed in the suspension studies. This was mainly due to the fact that we were monitoring the continuous release of dye in the microcolumn, whereas in suspension studies, we monitored the total dye that was accumulated for a certain period of time. The initial high fluorescence intensity can be due to the release of fluorescein that was entrapped near the surface of the porous silica microsphere and inner leaflet of the lipid bilayer. After a certain period of time, the fluorescence intensity became less due to the slow diffusion of dye from microsphere pores and the nearly complete removal of the dye from porous microspheres.

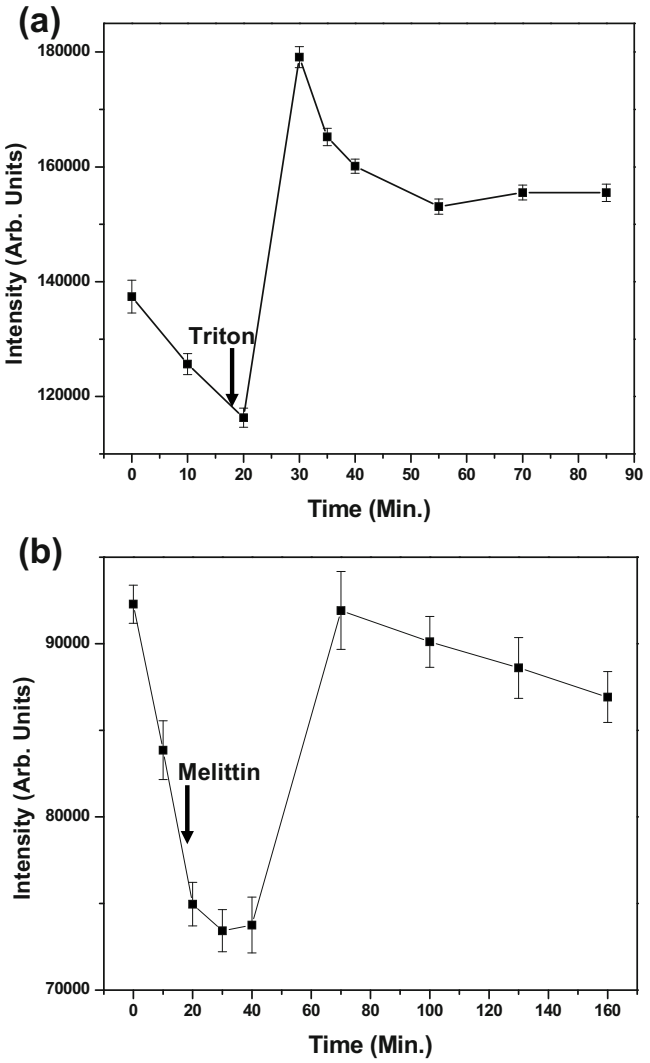

FIG. 10. Microfluidic membrane interaction analyses by release of compounds: (a) with 10% (w/v) Triton X-100 and (b) with 220 μM melittin. The flow rate is 1.2 μl/min. This figure is representative of three replicates.

3. Detection of interactions with SLBs on porous microspheres through the release of a bioactive compound

We aimed at demonstrating that our microfluidic system can be utilized to detect the release of nonfluorescent compounds upon disruption of SLBs on microspheres in packed microcolumns. Nonfluorescent compounds of interest might include highly bioactive compounds such as cofactors, catalysts, and ligands that can engender a significant downstream amplification mechanism. As a model, we chose biotin as a ligand with high biospecific activity. This was done in a microchannel that was designed to have two active segments of microspheres separated by a passive one (blank microspheres). In the first segment, the disruption of SLBs on microspheres took place, resulting in the release of the nonfluorescent compound, biotin. In the second segment, subsequent interactions of the released biotin occurred, which enabled its detection through the ostrich quenching-unquenching phenomena in fluorescein biotin-streptavidin interaction.[39] When a nonsaturating amount of fluorescein-conjugated biotin is bound to streptavidin, fluorescein interacts with *cis* biotin-binding pocket on streptavidin. This results in a quenching of its fluorescence intensity, which is known as ostrich quenching. The introduction of free biotin to this system displaces the fluorescein from the *cis* binding pocket, thus unquenching the fluorescence signal.[39]

In our study, as shown in Fig. 8(c), a segment of streptavidin microspheres (diameter=20 μm) coated with biotin that is conjugated to fluorescein is packed at the bottom of the microfluidic channel. Packed on top of that is a segment of blank silica (nonporous) microspheres followed by a segment of lipid-coated porous silica microspheres that encapsulate biotin. Our aim was to disrupt the SLB by Triton X-100 or melittin so as to release biotin, which, upon reaching the second segment, binds to streptavidin and displaces

fluorescein from the biotin-binding pocket on streptavidin, leading to unquenching, which is detectable as an increase in fluorescence intensity at that segment.

Figure 10(a) illustrates the detection of disruption of lipid membranes with Triton X-100. The initial decrease in fluorescence intensity was due to photobleaching, resulting from the exposure of the fluorescein-biotin segment to the Ar-ion laser.[11] To minimize the photobleaching, the microsphere segment was scanned only for 30 s at 10 min intervals. At 18 min, 10 μl of Triton X-100 (10% w/v) were injected. The flow rate was $\sim$1.2 μl/min and the dead volume from the injection point to the detection point was about 10 μl. As seen previously [Fig. 9(c)], initially, the porous microspheres released a large amount of entrapped biotin from porous microspheres, causing unquenching of biotin-conjugated fluorescein, which corresponds to the high fluorescence intensity observed at 30 min [Fig. 10(a)]. The presence of excess biotin can cause the dissociation of fluorescein-biotin from streptavidin; thus, the decrease in fluorescence intensity after 30 min may be due to the combined effects from photobleaching and the dissociation of some fluorescein-biotin from streptavidin. Figure 10(b) shows the data for the release of biotin as a result of melittin interaction with SLBs. 20 μl of melittin (220 μM) was injected into the microsphere packed microchannel at 18 min. The flow rate was maintained at $\sim$1.2 μl/min. The initial fluorescence intensity decrease is again due to photobleaching. The reading at 40 min indicated an increase in fluorescence intensity (as opposed to the expected decrease due to photobleaching), indicating unquenching of fluorescein. At this point, the frequency of laser scanning of the column was decreased from 10 to 30 min to prevent excess photobleaching. This was particularly a concern with melittin because we observed in the suspension studies that its disruption of bilayers caused a slow release; hence, photobleaching can have a significant effect on the detection of fluorescence intensity. At 60 min, we detected an increase in fluorescence due to the unquenching of fluorescein. The increase in fluorescence intensity caused by melittin was not as high as that observed with Triton X-100, which is in accordance with our suspension studies.

IV. CONCLUSIONS

In this work, we demonstrated the feasibility of using lipid-coated porous silica microspheres that encapsulate compounds as a platform for developing new chemical sensors for membrane-detergent and membrane-toxin interactions. We successfully encapsulated compounds into porous silica microspheres by forming a supported phospholipid bilayer that was stable for up to a month. We established that the SLBs on porous microspheres can endure experimental conditions necessary for their incorporation into packed microcolumns while maintaining the bilayer integrity and functionality.

We have demonstrated membrane interaction studies with SLBs on porous silica microspheres in suspensions and in microcolumns. We showed that the membrane interactions with detergents and membrane active proteins and peptides in microcolumns can be detected by (i) the release of a fluorescent dye and (ii) the release of compounds with high bioactivity by using biotin as a model. For proof-of-principal studies, we used Triton X-100, α-hemolysin, and melittin as membrane disruptors. We have further demonstrated the formation of stable SLBs containing negatively charged lipids on porous silica microspheres.

Further studies are needed to analyze the encapsulation efficiency of microspheres with different pore sizes and with different lipid formulations. The prototype microfluidic assays demonstrated here can be further refined by optimizing fluidic and detection systems. In principle, the methods described here can be used to study and detect the action of a wide variety of membrane active compounds. The microspheres with SLBs can be useful for a variety of high throughput screening methods.

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