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Enhancing Protein Stability by Adsorption onto Raftlike Lipid Domains

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Abstract: We demonstrate that the stability of adsorbed proteins can be enhanced by controlling the heterogeneity of the surface—by creating raftlike domains in a soft liposomal membrane. Recent work has shown that enzymes adsorbed onto highly curved nanoscale supports can be more stable than those adsorbed on flat surfaces with nominally the same chemical structure. This effect has been attributed to a decrease in lateral interenzyme interactions on a curved surface. Exploiting this idea, we asked if adsorbing enzymes onto "patchy" surfaces composed of adsorbing and nonadsorbing regions can be used to reduce lateral interactions even on relatively flat surfaces. We demonstrate that creating domains on which an enzyme can adsorb enhances the stability of that enzyme under denaturing conditions. Furthermore, we demonstrate that the size of these domains has a considerable effect on the degree of stability imparted by adsorption. Such biomimetic raft-inspired systems may find use in applications ranging from biorecognition to the design of novel strategies for the separation of biomolecules and controlling the interaction of multicomponent membrane-bound enzymes.

Introduction

Interfacing proteins with nanomaterials has gained considerable interest in recent times for applications ranging from biosensing to biorecognition, self-assembly, and the therapeutic delivery of proteins into cells.[1−4] As a result, numerous methods[5−8] have been explored to attach proteins onto a variety of nanomaterials including organic and inorganic nanotubes and nanoparticles. There has been an increasing emphasis on obtaining a fundamental understanding of the influence of nanomaterials on the structure and function of proteins. For instance, research groups have demonstrated that differences in nanoparticle size can strongly influence the secondary structure and activity of adsorbed proteins.[9,10] Roach et al.[11] and Hong et al.[12] reported the ability to control protein structure and function by tailoring the surface chemistry of nanoparticles. Recently, Asuri et al.[5,13] have demonstrated the ability of nanomaterials to stabilize proteins under harsh conditions to a greater extent than conventional flat supports.

These previous studies have primarily focused on proteins attached to "hard" nanomaterials. Fewer studies have been conducted to understand the interactions of proteins with soft nanomaterials such as liposomes and polymersomes.[14−16] Liposomes, also referred to as vesicles, are spherical baglike structures, with an aqueous core and an outer layer that is made up of a lipid bilayer, and can be considered as mimics of a cellular membrane. Recent studies suggest that cellular membranes are characterized by spatial variations in composition[17,18] and that the concentration of proteins, peptides, or other ligands in raftlike membrane domains may influence phenomena ranging from signal transduction in cells[17] to recognition in biomimetic

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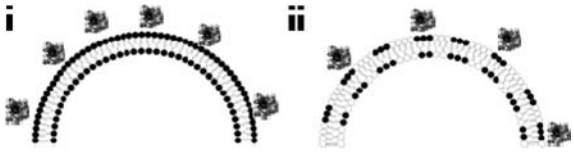
(1) Barone, P. W.; Baik, S.; Heller, D.; Strano, M. S. *Nat. Mater.* **2005**, *4*, 86–92.
(2) Chen, R. J.; Bangsaruntip, S.; Drouvalakis, K. A.; Kam, N. W. S.; Shim, M.; Li, Y. M.; Kim, W.; Utz, P. J.; Dai, H. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4984–4989.
(3) Graff, R. A.; Swanson, J. P.; Barone, P. W.; Baik, S.; Heller, D.; Strano, M. S. *Adv. Mater.* **2005**, *17*, 980–984.
(4) Kam, N. W. S.; Jessop, T. C.; Wender, P. A.; Dai, H. J. *J. Am. Chem. Soc.* **2004**, *126*, 6850–6581.
(5) Asuri, P.; Karajanagi, S. S.; Yang, H. C.; Yim, T. J.; Kane, R. S.; Dordick, J. S. *Langmuir* **2006**, *22*, 5833–5836.
(6) Wong, S.; Peng, X. *Adv. Mater.* **2009**, *21*, 625–642.
(7) Sun, Y.; et al. *Anal. Bioanal. Chem.* **2007**, *387*, 1565–1572.
(8) Li, Z.; Yub, W.; Sun, H.; Chen, J. *Mater. Res. Bull.* **2006**, *41*, 2268–2275.
(9) Asuri, P.; Bale, S. S.; Karajanagi, S.; Kane, R. S. *Curr. Opin. Biotechnol.* **2006**, *17*, 562–568.
(10) Vertegel, A. A.; Siegel, R. W.; Dordick, J. S. *Langmuir* **2004**, *20*, 6800–6807.
(11) Roach, P.; Farrar, D.; Perry, C. C. *J. Am. Chem. Soc.* **2006**, *128*, 3939–3945.
(12) Hong, R.; Fisher, N. O.; Verma, A.; Goodman, C. M.; Emrick, T.; Rotello, V. M. *J. Am. Chem. Soc.* **2004**, *126*, 739–743.
(13) Asuri, P.; Karajanagi, S. S.; Vertegel, A. A.; Dordick, J. S.; Kane, R. S. *J. Nanosci. Nanotechnol.* **2007**, *7*, 1675–1678.
(14) Ahmed, F.; Photos, P. J.; Discher, D. E. *Drug Dev. Res.* **2006**, *67*, 4–14.
(15) Discher, B. M.; Won, Y. Y.; Ege, D. S.; Lee, J. C. M.; Bates, F. S.; Discher, D. E. *Science* **1999**, *284*, 1143–1146.
(16) Discher, D. E.; Ahmed, F. *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323–341.
(17) Simons, K.; Ikonen, E. *Nature (London)* **1997**, *387*, 569–572.
(18) Brown, D. A.; London, E. *Annu. Rev. Cell Dev. Biol.* **1998**, *14*, 111–136.

Scheme 1. Soybean Peroxidase Adsorbed on (i) a Homogeneous Gel-Phase Liposome and (ii) "Raftlike" Domains in a Heterogeneous Liposome



systems.[19,20] These results further motivate our study of protein activity and stability on patchy liposomes.

Here, we report the preparation of stable and catalytically active liposome−enzyme conjugates from both homogeneous and heterogeneous liposomes. Moreover, we illustrate that membrane heterogeneity provides control over the adsorption and stability of adsorbed enzymes. Specifically, we demonstrate the selective adsorption of enzymes onto domains formed in heterogeneous membranes as well as the ability to enhance enzyme stability by adsorption onto raftlike domains (Scheme 1). We further demonstrate that the stability of adsorbed enzymes is influenced by the size of the domains in the liposomal membrane. This ability to influence protein function by tuning the heterogeneity of the underlying surface could have numerous applications in the field of biotechnology.

Methods

Preparation of Liposomes. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. To prepare homogeneous gel-phase cationic liposomes, a clean glass syringe was used to transfer 2 mg of 1,2-dipalmitoyl-3-trimethylammoniumpropane (DPTAP, $T_m = 43$ °C)[21] in chloroform to a glass vial that had been precleaned with chloroform and dried under a stream of nitrogen. Subsequently, chloroform was allowed to evaporate under an argon stream while the vial was constantly rotated by hand, resulting in formation of a thin lipid film on the wall of the glass vial. The residual chloroform was removed under vacuum for 4 h. The dried lipid mixture was then resuspended by adding 1 mL of 50 mM phosphate buffer containing 200 mM NaCl (pH 8.0) at 60 °C. The vial was then placed in a water bath at 60 °C overnight for rehydration, resulting in the formation of multilamellar vesicles. The multilamellar vesicles thus formed were then extruded at 60 °C with 21 passes through polycarbonate membranes (100 nm diameter pore size) by use of an Avanti miniextruder (Avanti Polar Lipids) to form small unilamellar vesicles (SUVs). Similarly, phase-separated liposomes were prepared by mixing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, $T_m = -19$ °C)[22] and DPTAP in a 3:1 molar ratio to a final total lipid weight of 8 mg. The chloroform was allowed to evaporate as described above, and the lipid film was dissolved in 50 mM phosphate buffer (pH 8.0) at a temperature of 60 °C. After the lipid was allowed to rehydrate overnight, the liposomes were extruded through a polycarbonate filter (100 nm diameter) at 60 °C as described above. The extruded liposome solution was then cooled either by placing the solution in a 4 °C cold room or by placing the extruded sample in a 1.4 L water bath that was cooled from 60 °C to ambient temperature over the course of 2.5 h in order to form phase-separated domains of different sizes.[23,24]

Measurement of Liposome Radius by Dynamic Light Scattering. Liposome radii were determined by use of a Protein Solutions MS800/12 apparatus. The incident beam was 824 nm polarized light at 90° to the detector. All samples were prefiltered before measurement with a 0.22 μm syringe filter.

Adsorption of Soybean Peroxidase onto Liposomes. Soybean peroxidase (SBP) was obtained from Sigma−Aldrich in powder form and used as received. Typically, SBP was dissolved in phosphate buffer (50 mM, pH 8.0) to give a stock solution at a final protein concentration of 1 mg/mL. This stock solution was diluted as required to conduct the experiments. For adsorption onto liposomes, 200 μL of SBP solution at the required concentration was mixed with 200 μL of a solution containing liposomes in a 1.5 mL Eppendorf tube and the tubes were left on a rocker platform for 2 h to ensure complete adsorption of SBP onto liposomes.

Determination of Saturation Coverage. The amount of enzyme adsorbed onto liposomes was determined by polyacrylamide gel electrophoresis (PAGE). Liposomes were exposed to freshly prepared solutions of SBP (10−300 μg/mL) for 2 h as described above, and the solutions were run in a 4−12% Tris−glycine gel at 150 V for ca. 30 min under native buffer conditions. The concentrations of the protein in the gel (the unbound protein) were determined by ImageJ analysis and used to determine the amount of SBP adsorbed onto liposomes.

Determination of Enzyme Activity. SBP catalyzes the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in the presence of H_2O_2 to form a soluble end product that can be read spectrophotometrically at 405 nm. The initial rates of SBP-catalyzed oxidation of ABTS were therefore determined by monitoring the increase in absorbance at 405 nm with an HTS 7000 Plus bioassay reader (Perkin-Elmer, Wellesley, MA). To measure enzyme stability under denaturating conditions, liposome−SBP conjugates and native SBP were incubated in buffer solutions containing 50% (v/v) methanol at room temperature. Aliquots were removed periodically and diluted such that the final methanol concentration was less than 1% (v/v). The initial enzyme rate was then measured at room temperature via the ABTS assay as described above.

Circular Dichroism Spectroscopic Studies. Unfolding of the SBP conjugates was also monitored by circular dichroism (CD) spectroscopy. The far-UV CD spectra (200−250 nm) of native SBP and liposome-SBP conjugates were recorded on an Olis DSM-10 CD instrument (Bogard, GA) at 20 °C using cylindrical quartz cuvettes with a 10 mm path length. In all measurements, the protein concentration was 50 μg/mL. At least three CD spectra were acquired for each sample. The spectra were then averaged and the α-helix content was calculated on the basis of mean residue ellipticity at 222 nm. For methanol denaturation studies, protein solutions were incubated in buffer solutions containing 50% methanol at room temperature and aliquots were taken periodically for data acquisition.

Visualization of Selective Protein Adsorption on Giant Unilamellar Vesicles. Giant unilamellar vesicles (GUVs) were prepared by the soft hydration method.[25] Briefly, a 75:24:1 mixture of DOPC, DPTAP, and, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) in chloroform was dried under a stream of argon to form a thin film at the bottom of a vial. The vial was then placed in vacuum for 4 h to remove any excess chloroform, yielding a mixture of lipids with a final weight of 2 mg. To this vial was added 1 mL of a 0.5 M aqueous sucrose solution at 60 °C, and the vial was kept at this temperature with no agitation overnight for rehydration. GUV formation was confirmed by the formation of a translucent layer near the top of the liquid. To this GUV suspension, 200 μL of a 0.5 mg/mL aqueous solution of fluorescein-conjugated soybean peroxidase were added, and the

(19) (a) Rai, P.; Saraph, A.; Ashton, R.; Poon, V.; Mogridge, J.; Kane, R. S. *Angew. Chem.* **2007**, *46*, 2207–2209. (b) Rai, P.; Vance, D.; Poon, V.; Mogridge, J.; Kane, R. S. *Chem.—Eur. J.* **2008**, *14*, 7748–7751.
(20) Webb, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 14462–14463.
(21) McKiernan, A. E.; Ratto, T. V.; Longo, M. L. *Biophys. J.* **2000**, *79*, 2605–2615.
(22) Clayden, N. *Phys. Chem. Chem. Phys.* **1999**, *1*, 4379–4382.
(23) Blanchette, C. D.; Lin, W.-C.; Ratto, T. V.; Longo, M. L. *Biophys. J.* **2006**, *90*, 4466–4478.
(24) Ratto, T. V.; Longo, M. L. *Biophys. J.* **2002**, *83*, 3380–3392.
(25) Battle, A.; Petrov, E.; Pal, P.; Martinac, B. *FEBS Lett.* **2009**, *583*, 407–412.

vial was placed on a rocker platform at 4 °C overnight. The GUVs were then imaged on a Zeiss LSM 510 confocal microscope by use of two-track excitation. DiI was excited at 544 nm and fluorescein at 488 nm. Z-stack images were taken at 1 μm intervals and combined to form a three-dimensional image of the GUV by use of the Zeiss LSM 510 META software.

Fluorescence Resonance Energy Transfer-Based Determination of Domain Size. Lipids were mixed in chloroform to the following compositions and a final overall lipid weight of 2 mg: 1.4% NBD-DOPE, 1.8% DiI, 25% DPTAP, and 71.8% DOPC. Liposomes were prepared as described above. We also prepared liposomes without DiI, with variations in DOPC concentration to keep total lipid amount and relative concentration of the other components unchanged. Each sample was heated to 60 °C for 1 h and then quenched to a temperature below the phase-transition temperature of DPTAP at one of two different rates. One set of samples was cooled at 4 °C for 1 h and the other was cooled by setting the temperature controller on the water bath to room temperature and allowing it to cool from 60 °C to room temperature over 2.5 h. The samples were then allowed to equilibrate at room temperature for 1 h prior to making fluorescence measurements.

In order to determine the degree of FRET between NBD-DOPE and DiI, 1 mL of liposome sample was placed in a quartz cuvette and fluorescence was measured with a Shimadzu fluorometer. The sample was excited at 340 nm and emission was read at 520 nm. The FRET efficiency was then calculated:

$$\varepsilon = 1 - \frac{I_{DA}}{I_D} \quad (1)$$

where I_{DA} represents the intensity of fluorescence in the presence of both donor (NBD-DOPE) and acceptor (DiI), while I_D represents the intensity of a sample containing only donor molecules (at the same concentration).

A numerical analysis was performed in order to establish the relationship between domain size and FRET efficiency. A program was written in Visual Basic that creates a grid representing the position of each lipid molecule in the bilayer. A phase-separated region was generated at the center of this grid, and the overall grid size was varied so that the ratio of donor to acceptor molecules in the simulation remained constant regardless of domain size. An identical secondary grid was created to represent the other leaflet of the liposome. Donor (NBD-DOPE) and acceptor (DiI) molecules were then randomly distributed in the fluid and gel phases for both leaflets. NBD-DOPE was assigned a partitioning fraction of 0.84 with a preference for the fluid phase as reported elsewhere,[26] while DiI was allowed to partition entirely into the gel phase.[26] The program then calculated the probability of energy transfer between each donor/acceptor pair within both grids:

$$\varepsilon = \frac{{R_0}^6}{{R_0}^6 + R^6} \quad (2)$$

where R is the distance between the two fluorophores and R_0 is the Forster radius, which is 5 nm for this system.[26] Emission from each fluorescence donor was initially assigned a value of unity; the probabilities of no FRET ($1 - \varepsilon$) for a donor with each sequential acceptor were then multiplied to obtain the final steady-state fluorescence intensity for each donor. The sum of these values for all donor molecules was divided by the total number of donor molecules in the simulation and used to obtain the overall FRET efficiency. This process was repeated for domain sizes ranging from 5 to 14 nm in radius and averaged over multiple simulations for each domain size to generate a calibration curve for FRET efficiency as a function of domain size.

Results and Discussion

Structure and Activity of SBP Adsorbed onto Homogeneous Liposomes. We first prepared homogeneous liposomes composed of the cationic lipid DPTAP, which is in the gel phase at room temperature. Characterization of the liposomes by dynamic light scattering (DLS) revealed that their average radius was 48.1 ± 15.2 nm. We next exposed the homogeneous DPTAP liposomes to the negatively charged protein, SBP (p*I* 4.1, solution pH 8.1, ca. 5 nm in diameter)[5] at concentrations ranging from 0 to 300 μg/mg DPTAP. As seen in Figure 1, SBP adsorbed strongly onto the DPTAP liposomes with a saturation loading of 162 μg/mg of DPTAP.

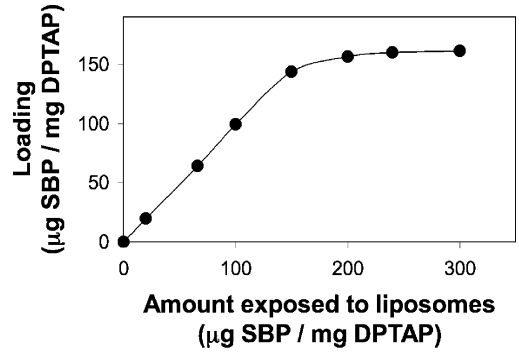


Figure 1. Plot of amount of SBP adsorbed versus amount of SBP exposed to liposomes (micrograms of SBP per milligram of DPTAP).

Next, we tested the activity of SBP adsorbed onto homogeneous DPTAP liposomes. To that end, SBP was adsorbed onto the liposomes at a concentration of 99 μg/mg of DPTAP (fractional surface coverage of ca. 0.6). SBP retained ca. 60% of its native activity when adsorbed onto the liposomes. This retention of activity compares well with that reported for SBP adsorbed onto other supports such as carbon nanotubes and graphite flakes.[5,13]

We also tested the stability of these liposome−SBP conjugates under denaturing conditions, in solutions containing 50% (v/v) of the denaturant methanol. The half-life ($\tau_{1/2}$) for DPTAP−SBP conjugates was ca. 103 min, a value that was significantly greater than that for the native enzyme in solution (ca. 26 min).

We used DLS to confirm that the liposomes were stable under these denaturing conditions. Characterization by DLS revealed that the radius of the liposomes after 3 h of exposure to a solution containing methanol (50% v/v) was 48.6 ± 13.2 nm, which is statistically indistinguishable from the value prior to methanol exposure.

Controlling SBP Adsorption on Domains in Heterogeneous Liposomes. We next wished to demonstrate the ability to pattern the adsorption of SBP onto specific domains in liposomes. To that end, we first made GUVs composed of the gel-phase cationic lipid DPTAP (phase transition temperature $T_m = 43$ °C),[21] the fluid-phase zwitterionic lipid DOPC ($T_m = -19$ °C),[22] and the dye DiI in a molar ratio of 25:74:1. To induce phase separation, the liposomes were heated to a temperature of 60 °C (higher than the T_m of both lipids) and then cooled by incubating at 4 °C, a temperature intermediate between the T_m values. We reasoned that this process would result in the formation of phase-separated domains:[23,24,27] gel-phase DPTAP-rich domains distributed in a continuous DOPC-rich fluid phase. Moreover, DiI partitions preferentially into domains enriched in gel-phase lipids, thereby enabling the domains to be visualized. We then exposed the GUVs to SBP−fluorescein conjugates, which were prepared by the reaction of SBP with the amine-reactive compound NHS-fluorescein (Thermo Scientific).

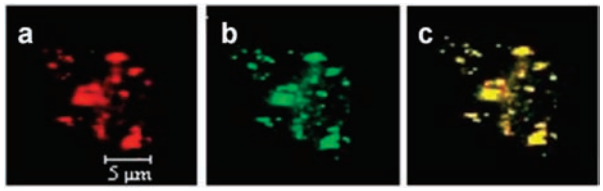


Figure 2. Selective adsorption of SBP−fluorescein conjugates onto DPTAP-rich domains in GUVs. Confocal micrographs are shown of (a) DiI partitioned into gel-phase domains of a GUV, (b) fluorescein-labeled SBP adsorbed on the same GUV, and (c) merged image demonstrating the selective adsorption of SBP on the gel-phase domains.

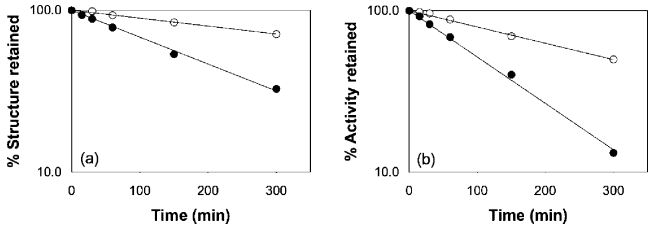


Figure 3. (a) Percent secondary structure retained vs time and (b) percent activity retained vs time for SBP adsorbed on homogeneous (●) and heterogeneous (○) liposomes following incubation in solutions containing 50% methanol.

Characterization by confocal microscopy (Figure 2) confirmed the highly selective binding of SBP onto DPTAP-enriched domains.

Effect of Liposome Heterogeneity on Enzyme Stability. Next, we compared the stability of SBP adsorbed onto homogeneous and heterogeneous liposomes (Scheme 1) in solutions containing 50% methanol. The homogeneous DPTAP liposomes and heterogeneous liposomes composed of DOPC and DPTAP in a molar ratio of 3:1 were prepared by extrusion through polycarbonate membranes as described above. To induce phase separation, the heterogeneous liposomes were cooled rapidly from 60 to 4 °C as described above. SBP was adsorbed onto the homogeneous and heterogeneous liposomes at a fractional surface coverage of 0.6.

Circular dichroism (CD) spectroscopy was used to characterize the secondary structure of SBP following incubation of the SBP−liposome conjugates in solutions containing 50% methanol. The α-helical content of SBP was calculated on the basis of the mean residual ellipticity at 222 nm:[10]

$$[\theta]_{222} = 100\theta M_w/clN_a \quad (3)$$

where $[\theta]_{222}$ represents the mean residual ellipticity at 222 nm, θ represents the measured ellipticity at 222 nm, c represents the protein concentration in grams per liter, l is the path length in centimeters, M_w is the molecular mass of SBP (37 000 Da),[28] and N_a is the number of amino acid residues. The measured α-helicity was then computed as $[\theta]_{222}/(-39\,500)$, with the value of $-39\,500$ deg cm^2 dmol^{-1} representing the value of $[\theta]_{222}$ for a peptide with 100% helicity. As seen in Figure 3a, characterization by CD spectroscopy revealed a slower rate of change of secondary structure for SBP adsorbed onto the heterogeneous liposomes as compared to SBP adsorbed onto homogeneous DPTAP liposomes. Consistent with these results, the $\tau_{1/2}$ for SBP adsorbed onto the heterogeneous liposomes was 297 min, representing a ca. 3-fold increase over that obtained on homogeneous DPTAP liposomes (Figure 3b). Moreover, this value of $\tau_{1/2}$ was significantly greater than that reported on other "hard" nanoscale supports such as carbon nanotubes, and nanoparticles.[5,9,13] Collectively, the data in Figure 3 clearly demonstrate that the adsorption of enzyme onto domains can result in a significant increase in stability relative to that on homogeneous surfaces.

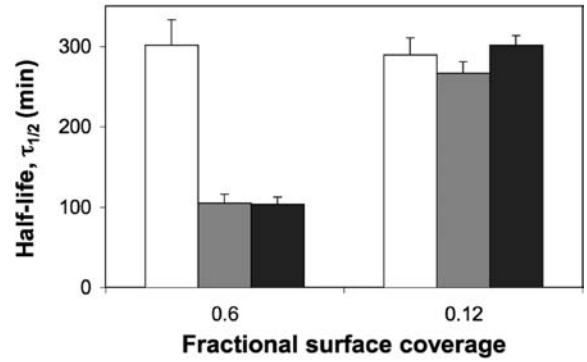


Figure 4. Stability under denaturing conditions for SBP adsorbed onto heterogeneous liposomes with small domains (white bars), heterogeneous liposomes with large domains (gray bars), and homogeneous liposomes (black bars).

Influence of Domain Size on Enzyme Stability. Next, we tested whether the size of the phase-separated domains in heterogeneous liposomes influences the stability of adsorbed SBP. The size of the phase-separated domains can be controlled by varying the cooling rate; faster cooling rates result in the formation of smaller domains compared to slower cooling rates.[23,24,27] Heterogeneous liposomes containing larger domains were generated by heating the liposomes to a temperature of 60 °C and then cooling them down at a slower rate by allowing the heated liposomes to reach room temperature over a period of 2.5 h. The $\tau_{1/2}$ of SBP adsorbed onto liposomes with larger domains (fractional surface coverage of 0.6) was 115 min, very similar to that found for SBP adsorbed onto homogeneous liposomes ($\tau_{1/2} = 103$ min) but significantly lower than that found on liposomes with smaller domains ($\tau_{1/2} = 297$ min) (Figure 4).

To better understand how domain size influenced enzyme stability, we used fluorescence resonance energy transfer (FRET) techniques to estimate the size of the phase-separated domains obtained at two different cooling rates. Liposomes composed of a mixture of DPTAP, DOPC, the fluorescent "donor" lipid NBD-DOPE (which partitions preferentially into fluid-phase domain), and the "acceptor" DiI were heated to 60 °C and cooled at two different rates as described above. Since NBD-DOPE and DiI act as a FRET pair, the donor (NBD-DOPE) fluorescence signal in the presence and absence of acceptor (DiI) can be used to determine FRET efficiency and therefore calculate the average domain size. Efficiencies were calculated by use of eq 1 (see Methods section for details). The FRET efficiency was 82% ± 2% and 45% ± 5% for the faster and slower cooling rates, respectively (Figure 5); higher FRET efficiency is expected for faster cooling rates, as the resulting smaller domains would result in smaller values of the average separation between the donor, NBD-DOPE, and the acceptor, DiI.

To relate these measured values of FRET efficiency to domain size, we carried out simulations (Figure 5). The accuracy of the simulations was validated by comparison with previously

(26) Loura, L.; Federov, A.; Prieto, M. *Biochim. Biophys. Acta* **2000**, *1467*, 101–112.
(27) de Almeida, R. F. M.; Loura, L. M. S.; Fedorov, A.; Prieto, M. *Biophys. J.* **2002**, *82*, 823–834.
(28) Kizhakkedathu, A.; Nazeerunnisa, M.; Behere, D. *J. Biol. Chem.* **2002**, *277*, 40717–40721.

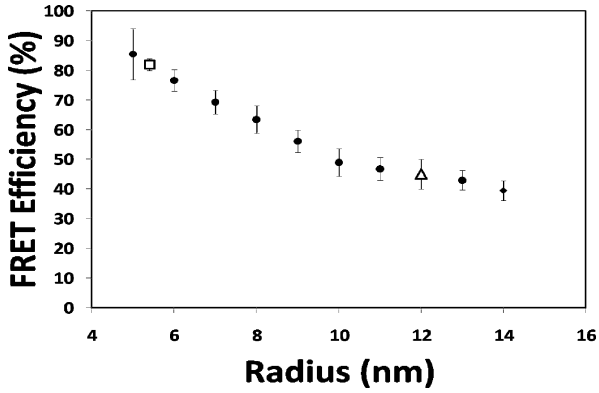


Figure 5. Relationship between radius of phase-separated domains and FRET efficiency. Results of computational analysis correlating FRET efficiency with domain radius (●). Also shown are experimentally measured FRET efficiencies for heterogeneous liposomes prepared by rapid cooling (□) and slow cooling (△).

reported models for the FRET-based estimation of domain size.[29,30] Based on the experimentally observed FRET efficiencies, the simulations predict average domain radii of 5.4 ± 0.23 nm and 12 ± 2.5 nm for the faster and slower cooling rates, respectively. A fractional surface coverage of 0.6 corresponds to an average number of SBP molecules per domain of 0.6 and 3, respectively, for the smaller and larger domains.

Influence of Surface Coverage on Enzyme Stability. Previous research has suggested that unfavorable "lateral" interactions between adsorbed proteins can influence protein stability under denaturing conditions.[5] When an adsorbed protein unfolds at high surface coverages, its residues can interact with residues on neighboring adsorbed proteins. These lateral interprotein interactions can promote the loss of native structure and hence result in a greater rate of protein deactivation.[5,13] A difference in the extent of unfavorable lateral interprotein interactions would explain the enhanced protein stability when the average number of adsorbed SBP molecules per domain is less than 1 (Figure 6a,c,e).

To further explore the validity of this hypothesis, we tested the stability of SBP adsorbed onto heterogeneous and homogeneous liposomes at a low fractional surface coverage of 0.12 (Figure 6b,d,f). At this lower surface coverage of SBP, both large and small domains would contain less than one adsorbed SBP molecule on average. We therefore hypothesized that these low surface coverages would result in the suppression of lateral interactions and the rate of deactivation on all liposomes (Figure 6b,d,f). Consistent with our hypothesis, the stability of adsorbed SBP was similar on all three liposomes at a low fractional coverage of 0.12 (Figure 4). Furthermore, the $\tau_{1/2}$ values on all three liposomes at this low surface coverage were similar to those obtained for SBP adsorbed onto small domains in a heterogeneous liposome at a surface coverage of 0.6 (Figure 4). These results suggest that segregating proteins on phase-separated domains can provide control over the extent of interprotein interactions and significantly enhance protein stability.

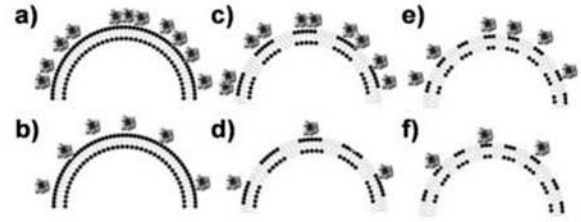


Figure 6. Schematic illustrating SBP adsorbed at high and low coverage on (a, b) homogeneous liposomes, (c, d) heterogeneous liposomes with large domains, and (e, f) heterogeneous liposomes with small domains.

Conclusions

We demonstrate the preparation of novel catalytically active and stable enzyme−liposome conjugates by employing homogeneous and heterogeneous liposomes as nanoscale supports for the adsorption of SBP. We used membrane heterogeneity to pattern the adsorption of the enzyme. Our results demonstrate the ability to control protein stability by controlling the heterogeneity of the underlying soft material. We have applied the concept of lipid rafts in a biotechnological context by using the segregation of proteins in phase-separated domains to influence the nature and extent of protein−protein interactions. Such biomimetic raft-inspired systems may find use in applications ranging from biorecognition to the design of novel strategies for the separation of biomolecules and controlling the interaction of multicomponent membrane-bound enzymes.

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(29) Kiskowski, M. A. *Biophys. J.* **2007**, *92*, 3040–3051.
(30) Brown, A. C.; Towles, K. B.; Wrenn, S. P. *Langmuir* **2007**, *23*, 11180–11187.