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Enhancing Protein Stability by Adsorption onto Raft-like 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 raft-like 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 inter-enzyme interactions on a curved surface. Exploiting this idea, we asked if adsorbing enzymes onto “patchy” surfaces composed of adsorbing and non-adsorbing 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 multi-component 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 the 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.

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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 bag-like 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 raft-like membrane domains may influence phenomena ranging from signal transduction in cells¹⁷ to recognition in biomimetic 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 using 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 raft-like 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-Trimethylammonium-Propane (DPTAP, $T_m=43\text{ }^{\circ}\text{C}^{21}$) in chloroform to a glass vial that had been pre-cleaned with chloroform and dried under a stream of nitrogen. Subsequently, chloroform was allowed to evaporate under an argon stream while constantly rotating the vial by hand, resulting in the 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 re-suspended 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 re-hydration, 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) using an Avanti mini-extruder (Avanti Polar Lipids, AL) to form small unilamellar vesicles (SUVs). Similarly, phase separated liposomes were prepared by mixing 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC, $T_m = -19\text{ }^{\circ}\text{C}^{22}$) 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 allowing the lipid 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 by either placing the solution in a 4 °C cold room or by placing the

extruded sample in a 1.4 L water bath which was cooled from 60 °C to ambient temperature over the course of 2.5 hours in order to form phase separated domains of different sizes.^{23,24}

Measurement of Liposome Radius by Dynamic Light Scattering (DLS)

Liposome radii were determined using a Protein Solutions MS800/12 apparatus. The incident beam was 824 nm polarized light at 90° to the detector. All samples were pre-filtered 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 µg/mL – 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 using ImageJ analysis and used to determine the amount of SBP adsorbed onto liposomes.

Determination of Enzyme Activity

SBP catalyzes the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in the presence of H₂O₂ 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 the absorbance at 405 nm using an HTS 7000 Plus Bioassay Reader (Perkin-Elmer, Wellesley, MA). To measure enzyme stability under denaturing 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 using the ABTS assay as described above.

Circular Dichroism Spectroscopy 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 alpha-helix content was calculated on the basis of the

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

FRET-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 to DOPC concentration to keep total lipid amount and relative concentration of the other components unchanged. Each sample was heated to 60 °C for one hour 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 one hour 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 hours. The samples were then allowed to equilibrate at room temperature for one hour prior to making fluorescence measurements.

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

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

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 which 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 using the relation:

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

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 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 (pI 4.1, solution pH 8.1, ca. 5 nm in diameter⁵) at concentrations ranging from 0 to 300 $\mu\text{g}/\text{mg}$ DPTAP. As seen in Figure 1, SBP adsorbed strongly onto the DPTAP liposomes with a saturation loading of 162 $\mu\text{g}/\text{mg}$ 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 $\mu\text{g}/\text{mg}$ 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 hours 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, $T_m = 43$ °C²¹), the fluid phase zwitterionic lipid DOPC ($T_m = -19$ °C²²) 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). Characterization by confocal microscopy (Fig. 2) confirmed the highly selective binding of SBP onto the 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 °C 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 alpha-helical content of SBP was calculated on the basis of the mean residual ellipticity at 222 nm $[\theta]$ using equation 3.¹⁰

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

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 g / L, l is the path length in cm, M_w is the molecular weight of SBP (37,000 Da)²⁸ and N_a is the number of amino acid residues. The measured alpha-helicity was then computed as $[\theta]_{222} / (-39500)$, with the value of $-39500^\circ \text{ cm}^2 \text{ dmol}^{-1}$ representing the value of $[\theta]_{222}$ for a peptide with 100% helicity. As seen in Fig. 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 homogenous 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 (Fig. 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 Figures 3a and 3b clearly demonstrate that the adsorption of enzyme onto domains can result in a significant increase in stability relative to that on homogenous surfaces.

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) (Fig. 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 the two different cooling rates. Liposomes composed of a mixture of DPTAP, DOPC, the fluorescent “donor” lipid NBD-DOPE which partitions preferentially into fluid phase domains, 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 using equation 1 (see methods section for details). The FRET efficiency was $82 \pm 2\%$ and $45 \pm 5\%$ for the faster and slower cooling rates, respectively (Fig. 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 (Fig. 5). The accuracy of the simulations was validated by comparison with previously 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 inter-protein 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 inter-protein interactions would explain the enhanced protein stability when the average number of adsorbed SBP molecules per domain is less than one (Fig. 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 (Fig. 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 (Fig. 6 b,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 (Fig. 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 (Fig. 4). These results suggest that segregating proteins on phase-separated domains can provide control over the extent of inter-protein interactions and significantly enhance protein stability.

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 multi-component membrane-bound enzymes.

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Scheme 1.

Soybean peroxidase adsorbed on (i) a homogeneous gel phase liposome and (ii) “raft-like” domains in a heterogeneous liposome.

Figure 1.
Plot of amount of SBP adsorbed versus amount of SBP exposed to liposomes (µg SBP/mg DPTAP).

Figure 2. Selective adsorption of SBP-fluorescein conjugates onto DPTAP-rich domains in GUVs. Confocal micrograph 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 (dark circle), and heterogeneous (open circle) liposomes following incubation in solutions containing 50% methanol.

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

Figure 5. Relationship between radius of phase-separated domains and FRET efficiency. The black circles represent the results of computational analysis correlating FRET efficiency with domain radius. The open square and the open triangle represent experimentally measured FRET efficiencies for heterogeneous liposomes prepared by rapid cooling and slow cooling, respectively.

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