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Spectroscopic Characterization of Structural Changes in Membrane Scaffold Proteins Entrapped within Mesoporous Silica Gel Monoliths

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**ABSTRACT**

The changes in orientation and conformation of three different membrane scaffold proteins (MSPs) upon entrapment in sol-gel derived mesoporous silica monoliths were investigated. MSPs were examined in either a lipid-free conformation or a lipid-bound conformation, where the proteins were associated with lipids to form nanolipoprotein particles (NLPs). NLPs are water soluble, disk-shaped patches of lipid bilayer that have amphiphilic MSPs shielding the hydrophobic lipid tails. The NLPs in this work had an average thickness of 5 nm and diameters of 9.2 nm, 9.7 nm, and 14.8 nm. We have previously demonstrated that NLPs are more suitable lipid-based structures for silica gel entrapment than liposomes due to their size compatibility with the mesoporous network (2-50 nm) and minimally altered structure after encapsulation. Here we further elaborate on that work by using a variety of spectroscopic techniques to elucidate whether or not different MSPs maintain their protein-lipid interactions after encapsulation. Fluorescence spectroscopy and quenching of the tryptophan residues with acrylamide, 5-DOXYL-stearic acid, and 16-DOXYL-stearic acid was used to determine MSP orientation. We also utilized fluorescence anisotropy of tryptophans to measure the relative size of NLPs and MSP aggregates after entrapment. Finally, circular dichroism spectroscopy was used to examine the secondary structure of the MSPs. Our results showed that after entrapment,

all of the lipid-bound MSPs maintained orientations that were minimally changed and indicative of association with lipids in NLPs. The tryptophan residues appeared to remain buried within the hydrophobic core of the lipid tails in the NLPs and appropriately spaced from the bilayer center. Also after entrapment, lipid-bound MSPs maintained a high degree of  $\alpha$ -helical content – a secondary structure associated with protein-lipid interactions. These findings demonstrate that NLPs are capable of serving as viable hosts for functional integral membrane proteins in the synthesis of sol-gel derived bio-inorganic hybrid nanomaterials.

**KEYWORDS:** membrane scaffold protein, nanolipoprotein particle, mesoporous silica, tryptophan fluorescence, biohybrid material, protein-lipid interactions

## **INTRODUCTION**

The interface between biological species and inorganic materials serves as a platform for the development of many unique and interesting biomaterials. Encapsulation of functional proteins within mesoporous silica has been of particular interest to a wide variety of research areas over the last several decades due its extensive applications,<sup>1-2</sup> as well as the ability of silica to protect the entrapped protein from denaturation and proteolytic hydrolysis.<sup>3-4</sup> More recently, there has been increasing interest in entrapment of integral membrane proteins (IMPs) to synthesize biomaterials for applications such as biosensing, high throughput drug screening, affinity chromatography, and bioreaction engineering.<sup>5-7</sup> This is due to the various functionalities of IMPs, which includes enzymatic, transport, and receptor-ligand-binding activity.<sup>8</sup> IMPs, however, require a biological membrane (i.e. amphipathic lipid bilayer) into which it can insert to maintain an active tertiary conformation.<sup>8-9</sup> It is well known that the lack of a proper biological membrane or disruption of a lipid membrane bearing an IMP can result in inactivation

of the IMP.<sup>10-13</sup> Therefore, it is crucial to develop a bio-membrane/sol-gel architecture such that the biological membrane host structure is minimally altered upon encapsulation.

To ensure viable incorporation of biological membranes, a sol-gel route must be biologically friendly. The desire for protein entrapment has served as an impetus for development of biocompatible sol-gel methods. Research groups headed by Bright, Friedman, Kostic, and Brennan began examining properties of several water soluble proteins entrapped in silica gel monoliths during the 1990s.<sup>14-17</sup> This eventually led to techniques that included the use of additives, such as glycerol and sugar to alter the hydration of the protein.<sup>18-19</sup> However, this approach did not address the presence of elevated alcohol concentrations, which is problematic in biological systems. Typical sol-gel synthesis utilizes alkoxysilane precursors, such as tetramethylorthosilicate (TMOS) or tetraethylorthosilicate (TEOS). Upon hydrolysis of these precursors, methanol and ethanol are released from TMOS and TEOS, respectively.<sup>20</sup> Sufficiently high concentrations of alcohol are capable of denaturing proteins or causing lipid bilayers to interdigitate (i.e. become thinner).<sup>21-22</sup> The latter would directly affect the conformation of any IMPs being supported by the lipid bilayer. Brennan's group developed biocompatible sol-gel alkoxysilane precursors bearing sugar moieties and/or glycerol in place of alcohol groups.<sup>23-24</sup> This approach led to direct reduction of alcohol liberated during hydrolysis reactions, as well as the prevention of the additives being leached from the gel. They would later apply this sol-gel architecture to examine entrapment of liposomes as biological membrane hosts for IMPs and found that the liposomes ruptured after several days, as opposed to immediately when using the precursor TEOS.<sup>25</sup> The significantly different scale of liposomes (100 nm) in comparison to the porous structure (2-50 nm) of silica gels, in addition to the rather fragile

capsule-like structure of liposomes, may have played an important role in the eventual disruption of entrapped liposomes.

We propose use of nanolipoprotein particles (NLPs) as biological membrane hosts inside of mesoporous silica. NLPs are disk-shaped patches of lipid bilayer that have amphiphilic membrane scaffold proteins (MSPs) in contact with and shielding the hydrophobic lipid tails around the outer periphery of the particle, thus making the entire particle water-soluble. NLPs have an average thickness of 5 nm and have a tunable diameter that ranges from 10-25 nm based on the types of MSPs used.<sup>26</sup> The aqueous processing conditions of the sol-gel method make it amenable to incorporation of water-soluble NLPs. Also, the pore size distribution of the silica gel, 5-50 nm, provides a compatible size match for NLPs. In 2014, we first reported the stability of NLPs entrapped within mesoporous silica, where it was demonstrated that NLPs served as more viable structures than liposomes upon entrapment in silica gel.<sup>27</sup> To date, that was the first and only publication describing entrapment of NLPs in mesoporous silica gel monoliths. We performed detailed analysis of the lipid phase behavior of the NLP within the particles to find that it was minimally modulated upon entrapment in comparison to the phase behavior for lipids in liposomes, and that liposomes were undergoing significant changes in size in comparison to NLPs. This behavior was observable for a time period in excess of one month. The conformation of the particular MSP used in that work, referred to as MSP-3 in this work was also evaluated. We found that the MSP maintained a conformation where it perhaps continued to interact with the lipids after silica gel encapsulation (i.e. a lipid-bound conformation) for at least one week. The analysis performed for the MSP, however, only provided insight for the structure of the protein, rather than insight to the orientation of the MSP and elucidation as to whether or not the protein-lipid interactions truly remained intact.

In this work, we investigate the stability of the protein-lipid interactions in NLPs upon entrapment in mesoporous silica to determine whether or not a variety of different MSPs maintain a lipid-bound conformation. The encapsulation was performed using a simple sol-gel processing method for TMOS that utilizes rotary evaporation to remove the majority of methanol - a method first presented by Ferrer *et al.*<sup>28</sup> and previously used for entrapment of liposomal species.<sup>29</sup> We examined NLPs formed with three different types of Apolipoprotein AI derived MSPs, each having different chain lengths. These MSPs were initially synthesized and made commercially available by the research group of Stephen G. Sligar.<sup>30-31</sup> The orientations of the MSPs were determined via fluorescence spectroscopy and fluorescence quenching of tryptophan residues with acrylamide and two different nitroxide-spin-labeled stearic acids. Fluorescence anisotropy of the tryptophan residues was also used for determining the relative size of lipid-free and lipid-bound MSPs inside of the silica gel. Lastly, circular dichroism spectroscopy was used with two of the three MSPs to determine their secondary structure in lipid-free and lipid-bound states. We found that the MSPs maintained protein-lipid interactions within the NLPs upon encapsulation in a manner such that the interactions were minimally altered from that in solution. In combination with our previous work, these results strongly indicate that NLPs maintain their structure upon entrapment in silica gel.

## **MATERIALS AND METHODS**

**Materials.** Lyophilized MSP1D1 (MSP-1) and MSP1D1ΔH5 (MSP-2) were purchased from Cube Biotech, Inc., while lyophilized MSP1E3D1 (MSP-3) was purchased from Sigma Aldrich, Inc. MSP-1, MSP-2, and MSP-3 had chain lengths of 217 amino acids (25.3 kDa), 184 amino acids (21.5 kDa), and 277 amino acids (32.6 kDa), respectively. All MSPs were purchased with histidine tags attached to their N-termini. Tetramethyl Orthosilicate (TMOS) ( $\leq 99\%$ ),

Acrylamide ( $\geq 99\%$ ), Sodium Chloride ( $\geq 99\%$ ), Sodium Cholate ( $\geq 99\%$ ), Imidazole ( $\geq 99\%$ ), N-Acetyl-L-tryptophanamide, 5-DOXYL-stearic acid free radical (5-DOXYL), and 16-DOXYL-stearic acid free radical (16-DOXYL) were also purchased from Sigma Aldrich, Inc. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased in chloroform from Avanti Polar Lipids, Inc. Tris(hydroxymethyl)aminomethane (Tris, MB Grade) was purchased from USB Corporation, while Hydrochloric Acid (12.1 M), Sodium Phosphate Dibasic Heptahydrate, and Sodium Fluoride were purchased from Fischer Scientific, Inc. Ni-NTA agarose used for NLP purification was purchased from 5 PRIME, Inc. All of the water used for these experiments was purified in a Barnstead Nanopure System (Barnstead Thermolyne, Dubuque, IA) and had a resistivity of 17.9 M $\Omega$ •cm or greater.

**Synthesis of NLPs.** NLPs bearing MSP-1, MSP-2, and MSP-3 as scaffold proteins were used for tryptophan fluorescence quenching and anisotropy experiments, as well as far UV circular dichroism experiments. They were prepared as previously described<sup>27</sup> with modifications. Briefly, for a single preparation 5 mg of DMPC stock was dried in a glass conical vial under mild vacuum for at least 4 hours then rehydrated in a buffer consisting of 40 mM sodium cholate, 20 mM Tris, 100 mM NaCl (pH 7.4) and incubated at 37°C for 30 minutes. MSP-(1, 2, or 3) was then added to the mixture such that the mass ratio of lipid:protein was 5:2 for MSP-1 and MSP-2, and 5:1 for MSP-3. Next, the solubilized lipid/protein mixture was incubated at 4°C for 20 minutes, then 37°C for 20 minutes. This was repeated two additional times. Afterward, the mixture was transferred to a 10,000 MWCO dialysis cartridge (Thermo Scientific, Rockford, IL) and dialyzed against 20 mM Tris, 100 mM NaCl buffer (pH = 7.4) at 4°C for 48 hours at 200X the sample volume and underwent three buffer exchanges during this time period. From that point on, the rest of the procedure was performed identically to that of the aforementioned

protocol, where the resulting NLP mixture was purified using Ni-NTA agarose, eluted with imidazole based buffer, and dialyzed again for 24 hours to remove imidazole, resulting in purified NLPs in 20 mM Tris, 100 mM NaCl buffer (pH = 7.4).

**Entrapment of NLPs in Silica Gel.** NLP entrapment was performed as previously described<sup>27</sup> with slight modification. In a typical preparation, 7.6 mL of TMOS was combined with 5.6 mL of 0.002 M HCl (in water) in a beaker submerged in an ice bath, then subjected to probe tip sonication for 15 minutes. Afterward, rotary evaporation (340 mbar reduced pressure, 50°C) was used for 20 minutes to remove methanol formed during the hydrolysis reactions. The mixture was then passed through a 0.45 µm filter, resulting in roughly 4 mL of translucent silica sol. The sol was then combined with 20 mM Tris, 100 mM NaCl Buffer (pH 7.4) and an NLP sample of choice (1 part sol, 1 part buffer, 1 part sample; 3 equal volume portions) and allowed to gel in a methacrylate cuvette. Gelation typically occurred within 5-10 minutes. Gel volumes were approximately 2.5 mL. For samples used in quenching experiments, quenchers were added to the NLP sample before being combined with the sol. Lipid-free MSPs were encapsulated in an identical manner. All gelled samples contained approximately 5 v/v% methanol. The silica gel was confirmed to be mesoporous, containing pore sizes in the range of 2-50 nm via N<sub>2</sub> adsorption (Supporting Information).

**Tryptophan Accessibility to Solvent Determination via Acrylamide Quenching.** The relative accessibility of a tryptophan residue to solvent can be determined by using the Stern-Volmer relationship<sup>32-33</sup> (Equation1), where  $F_0$ ,  $F$ ,  $K_{SV}$ , and  $[Q]$  are the fluorescence intensity in the absence of quencher, fluorescence intensity in the presence of quencher, Stern-Volmer constant, and quencher concentration, respectively.

$$(1) \quad \frac{F_0}{F} = K_{SV}[Q] + 1$$



The Stern-Volmer relationship shows that the inverse of the relative fluorescence intensity is linearly proportional to the concentration of quencher used. Therefore, higher values of  $K_{SV}$  indicate that a tryptophan residue is more exposed to water (i.e. more efficiently quenched), while a lower  $K_{SV}$  value indicates that a tryptophan residue is less exposed to water (i.e. less efficiently quenched). The quencher used in these experiments was acrylamide, which is water soluble.

For solution quenching, samples from stock solutions of lipid-free and lipid-bound MSP-(1,2, or 3) were diluted to 4  $\mu$ M final MSP concentration (2.5 mL total volume) in a methacrylate cuvette and titrated with a stock solution of 4 M acrylamide in 25  $\mu$ L increments until a final concentration of approximately 0.16 M acrylamide was reached in the sample. For gel quenching, 5 samples for each type of lipid-free and lipid-bound MSP (30 different samples total) were created with varying acrylamide concentrations (0 – 0.16 M acrylamide) and an MSP concentration of 4  $\mu$ M. Gel emission spectra were corrected by performing control experiments with N-Acetyl-L-tryptophanamide (Supporting Information).

For all samples, tryptophan was excited at 295 nm and emission spectra were recorded from 300 – 450 nm. Each plot was regressed to remove noise and determine the emission spectra's maximum intensity and critical wavelength (Supporting Information). The maximum intensity from each emission scan with acrylamide present was normalized by the maximum intensity of the corresponding emission scan in which acrylamide was not present and used to fit Equation 1. All measurements were carried out on a Perkin Elmer LS 55 Fluorescence Spectrometer equipped with a PTP-1 Fluorescence Peltier System (PerkinElmer, Inc., Waltham, MA). Each measurement was performed in triplicate at  $22 \pm 1^\circ\text{C}$ . A scan speed of 180 nm/min was used and 5.0 nm slit-widths were used for both the excitation and emission monochromators.

**Tryptophan Position Determination via Parallax Analysis.** The parallax method was used to estimate the average position of tryptophan residues in the 3 MSPs when bound to lipids in NLPs. Due to the height difference between the nitroxide spin label in the 5-carbon and 16-carbon positions, 5-DOXYL and 16-DOXYL quench tryptophan in the MSP belt with different efficiencies.<sup>34-35</sup> The height of a tryptophan from the center of a bilayer is given by Equation 2.<sup>36-37</sup>

$$(2) Z_{eff} = L_{cd} - \frac{\ln\left[\frac{(F_s/F_o)^2}{(F_d/F_o)}\right]/\pi C - 2L_{ds}^2 + 4L_{cd}^2}{4(L_{ds} + L_{cd})}$$

$Z_{eff}$ ,  $L_{cd}$ , and  $L_{ds}$  are the effective tryptophan height, length between bilayer center and deep quencher, and length between the deep quencher to shallow quencher, respectively. In this work, 5-DOXYL is the shallow quencher, while 16-DOXYL is the deep quencher. Values for  $L_{cd}$  and  $L_{ds}$  were estimated for our system to be 0.82 Å and 11.33 Å (Supporting Information).  $F_s/F_o$  and  $F_d/F_o$  represent the relative tryptophan emission intensities in samples containing 5-DOXYL and 16-DOXYL, respectively.  $C$  is the concentration of DOXYL per area of lipid bilayer. This was estimated using the measured concentrations of the scaffold proteins and solution size of NLPs (Supporting Information). Equation 2 accounts for quenching of tryptophan from DOXYL present in both lipid leaflets (i.e. trans-bilayer quenching).

For solution quenching, a 2.5 mL NLP sample in a methacrylate cuvette (4 µM MSP) was titrated with a 10 mM stock solution of either 5-DOXYL or 16-DOXYL in ethanol. Titrations were performed in 3 µL increments until the final DOXYL concentration was 15 µM in the sample. For gel, multiple samples were created for each type of MSP (three each for MSP-1,2 and two for MSP-3). Each sample contained a different concentration of either 5-DOXYL or 16-DOXYL (0 – 15 µM) and 4 µM MSP.

For all samples, tryptophan was excited at 295 nm and emission spectra were recorded from 300 – 450 nm. Each plot was regressed to remove noise and determine the emission spectra's maximum intensity and emission wavelength (Supporting Information). The maximum intensity from each emission scan with DOXYL present was normalized by the maximum intensity of the corresponding emission scan in which DOXYL was not present and used to fit Equation 2. All measurements were carried out on a Perkin Elmer LS 55 Fluorescence Spectrometer equipped with a PTP-1 Fluorescence Peltier System (PerkinElmer, Inc., Waltham, MA). Each measurement was performed in triplicate at  $22 \pm 1^\circ\text{C}$ . A scan speed of 180 nm/min was used and 5.0 nm slit-widths were used for both the excitation and emission monochromators. No quenching was observed for lipid-free MSPs over the DOXYL concentration ranges used, as there were no lipid bilayers present into which DOXYL molecules could partition.

**Fluorescence Anisotropy of Tryptophan in MSPs.** Anisotropy values ( $r$ ) are a measure of the relative rotational speed of a fluorescent molecule.<sup>38</sup> It is calculated as the difference in fluorescence intensity of emitted parallel and perpendicular polarized light normalized by the entire intensity of light emitted, as shown in Equation 3.

$$(3) \quad r = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}}$$

Solution and gel samples containing 4  $\mu\text{M}$  MSP in methacrylate cuvettes were measured for their anisotropy values using a FluoroLog-3 FL3-22 Spectrophotometer (Horiba, Ltd., Kyoto, Japan). An excitation wavelength of 295 nm was used for all samples, while the emission wavelength depended on the sample, varying from 340 nm – 349 nm. An excitation slit-width of 5.0 nm and an emission slit-width of 2.5 nm was used. All anisotropy values were measured at  $22 \pm 1^\circ\text{C}$  and were the average of 10 anisotropy measurements.

**Circular Dichroism of MSPs.** Circular dichroism (CD) spectra of MSPs was performed as previously described.<sup>27</sup> Briefly, lipid-free MSPs and lipid-bound MSPs in 20 mM Tris, 100 mM NaCl buffer (pH 7.4) were exchanged into 25 mM Phosphate, 100 mM NaF buffer (pH 7.4) via dialysis. CD spectra were obtained using a Jasco J-715 spectropolarimeter (JASCO Easton, MD) with a scan range of 190 nm – 260 nm at room temperature in a demountable close-ended far UV 1 mm path length quartz cuvette for gel samples, and a 1 mm path length open end quartz cuvette for solution samples. CD spectra were normalized by converting the ordinate axis to molar ellipticity.<sup>39</sup> Alpha-helical content was determined as described elsewhere.<sup>27, 39</sup> The concentration of MSP in solution samples was approximately 0.2 mg/mL, while the concentration in silica gel was samples approximately 0.1 mg/mL.

## **RESULTS AND DISCUSSION**

Nanolipoprotein particles (NLPs) are self-assembled structures consisting of phospholipids and membrane scaffold proteins (MSPs), and were chosen to be investigated as potential hosts for integral membrane proteins due to their size compatibility with mesoporous silica. Though no direct analysis of integral membrane proteins was performed in this work, the integrity of membrane scaffold protein-lipid interactions in NLPs was examined. This is important because maintaining the structural integrity of a biological host (i.e. NLPs) would be crucial for its ability to keep integral membrane proteins folded correctly in future work. Lipid-free MSPs and MSPs bound to the lipid DMPC were examined in both aqueous solution and mesoporous silica gel for a variety of different analyses. The overall fluorescence emission and quenching of tryptophan residues within the MSPs via acrylamide or nitroxide-spin-labeled stearic acid was investigated, as well as fluorescence anisotropies of these same residues. In addition, the circular dichroism spectra of MSPs were recorded and interpreted. All of this

analysis was performed to elucidate the structural and conformational changes of MSPs upon entrapment in TMOS derived, mesoporous silica. Three different MSPs, each derived from the 10 helical regions of Apolipoprotein AI, were used in this work; MSP-1, MSP-2, and MSP-3 formed NLPs having discoidal diameters of 9.7 nm, 9.2 nm, and 14.8 nm, respectively (Supporting Information). The structure of each of these MSPs is depicted in Figure 1, where it is shown that each MSP contains tryptophan residues (W) in different positions along the length of the protein belt. These proteins were chosen to provide variability among the positioning and overall solvent accessibility of the tryptophan residues.

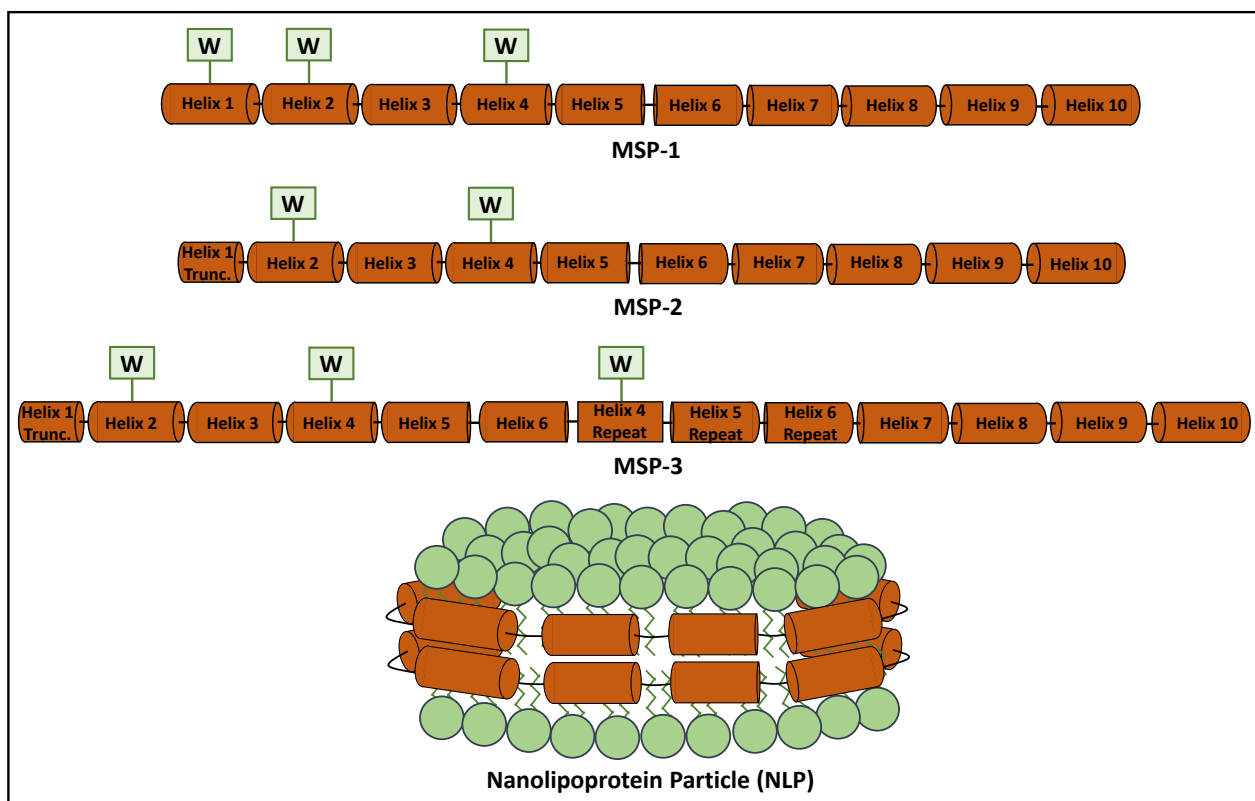


Figure 1: Sequences of the MSPs used and the location of tryptophan residues (W) in each one, with the N-terminus located on the left and the C-terminus located on the right. Below is the general structure of a Nanolipoprotein Particle (MSPs bound to lipids).

Tryptophan fluorescence emission is sensitive to its microenvironment. The maximum emission wavelength is known to undergo a blue-shift upon relocation to an environment with decreased polarity<sup>40</sup> (e.g. going from an aqueous environment to the tail region of lipid bilayers). The magnitude of this shift for a lipid-free to lipid-bound transition in Apolipoprotein A-I has been observed to be on the order of 2-3 nm.<sup>41</sup> This shift was reproducibly observed for all three of the MSPs utilized in this work, as illustrated in Table 1. MSP-1, MSP-2, and MSP-3 exhibited lipid-free to lipid-bound blue-shifts of 2 nm, 3 nm, and 1 nm, respectively, in solution, while displaying blue-shifts of 2 nm, 5 nm, and 4 nm in silica gel. This indicates that the MSPs are interacting with DMPC such that they remain oriented towards the carbon tails, even after entrapment. It is worth noting that after entrapment, the blue-shifts for MSP-2 and MSP-3 (5 nm and 4 nm, respectively) were of larger magnitudes than the blue-shift for MSP-1 (2 nm). The larger blue-shifts for MSP-2 and MSP-3 were determined to be statistically significant (Supporting Information). This can perhaps be attributed to the longer Helix 1 and presence of a Helix 1 tryptophan in MSP-1, and its absence in MSP-2 and MSP-3 (Figure 1). MSP-1 has been shown to exhibit dynamic behavior near its N- and C-termini when bound to lipids (i.e folded into NLPs) in solution.<sup>42</sup> This dynamic behavior would be associated with lack of defined structure in these regions. The blue-shifts observed for each MSP is the superimposed contribution from each tryptophan residue present in the protein. Therefore we speculate that for lipid-bound MSP-1 the Helix 1 tryptophan was relatively exposed to the aqueous environment before and after entrapment; this is captured in the statistically similar (Supporting Information) blue shift for lipid-bound MSP-1. We propose that the relatively shorter Helix 1 of MSP-2 and MSP-3 has less influence upon the lipid-bound MSP structure, allowing the confinement in the gel to impose more structure in lipid-bound MSP-2 and MSP-3, thus resulting in deeper

embedding of the Helix 2 and Helix 4 tryptophans (Figure 1) into the bilayer. This is observed as an increased blue-shift for MSP-2 and MSP-3. For elucidation of this theory, structural and dynamic characterization of each helix in these lipid-bound MSPs in a gel-environment would have to be performed. It can also be seen in Table 1 that the wavelengths of all gel-entrapped MSP emissions were lower in comparison to solution. This indicates that the environment within the pores of the silica were less polar than that of solution. This is perhaps due to the residual methanol content ( $\leq 5$  v/v%) that remained present after formation of the silica gel monoliths.

Table 1: Tryptophan fluorescence emission wavelengths of MSPs in solution and silica gel.

	Solution		Gel	
	Lipid-Free	Lipid-Bound	Lipid-Free	Lipid-Bound
<b>MSP-1</b>	349 nm	347 nm	346 nm	344 nm
<b>MSP-2</b>	348 nm	345 nm	345 nm	340 nm
<b>MSP-3</b>	346 nm	345 nm	344 nm	340 nm

Water-soluble acrylamide quenches the fluorescence emission from tryptophan in a proximity-dependent manner.<sup>32</sup> Therefore, the degree of quenching is tied to the local environment of the tryptophan residue – either solvent/water accessible or buried/hydrophobic. The goal of these acrylamide quenching experiments was to compare the quenching behavior of lipid-free and lipid-bound MSPs in solution, observe a difference, and verify that this difference was consistently observed with lipid-free and lipid-bound MSPs in gel. This would strongly suggest that lipid-protein interactions within the NLPs are maintained after gel entrapment. To compare solvent-accessibility of the tryptophan residues for lipid-free and lipid-bound forms of all three MSPs in solution or gel, we created Stern-Volmer plots of tryptophan fluorescence quenching vs. acrylamide concentration. The accessibility of acrylamide is encompassed in the Stern-Volmer constant ( $K_{SV}$ ) obtained through regression of the Stern-Volmer plots using the

Stern-Volmer relationship (Equation 1). Observing differences among the MSPs was expected, as it has been previously shown that tryptophan residues located in different positions along the scaffold protein belt exhibit different quenching behavior.<sup>34-35, 43</sup> Figure 2 shows the Stern-Volmer plots with regressed slopes,  $K_{SV}$ , for each of the different scaffold proteins in both solution and gel. In all six plots of Figure 2, lipid-free MSPs have larger slopes than the corresponding lipid-bound MSPs under the same conditions, even after gel encapsulation. This strongly suggests that for each of the three MSPs, the scaffold protein generally remains bound to the lipid after encapsulation. In Table 2, the values of  $K_{SV}$  for each MSP are displayed. For MSP-1 and MSP-2, the trends are similar, as lipid-free  $K_{SV}$  values decreased and lipid-bound  $K_{SV}$  values increased (but not significantly) upon entrapment in silica gel. For MSP-3, the  $K_{SV}$  values decreased for lipid-free samples, but also decreased (but not significantly) for lipid-bound samples once entrapped in the silica gel. The statistical significance of changes in  $K_{SV}$  was analyzed (Supporting Information) and it was determined that the difference in  $K_{SV}$  between a lipid-free to a lipid-bound MSP was significant for all samples. The statistical significance of the differences in  $K_{SV}$  values associated with solution and gel samples was also analyzed; lipid-free MSPs exhibited a significant change in  $K_{SV}$ , while lipid-bound MSPs did not. This indicates that the lipid-free MSPs could have undergone significant conformational changes upon entrapment, while the lipid-bound MSPs did not. MSPs are derived from Apolipoprotein AI, which forms a 4-helix bundle<sup>44</sup> and has relatively dynamic behavior<sup>42</sup> in the lipid-free state in solution. The silica gel entrapment could induce conformational changes in the lipid-free MSPs that alter their structures such that the tryptophan residues become less accessible to water/acrylamide. This speculation is corroborated by previous works where conformational changes in proteins that interact with porous silica surfaces have been observed.<sup>27, 45</sup> Table 2 also shows that for all



conditions, the trend  $K_{SV,MSP-1} > K_{SV,MSP-2} > K_{SV,MSP-3}$  holds. This is most likely due to the different location of tryptophan residues in each MSP and the dynamic behavior of the MSPs at the N-terminus;<sup>42</sup> MSP-1 contains two tryptophans near the N-terminus, while MSP-2 and MSP-3 only contain one. Also, MSP-3 contains a tryptophan in the center of the protein belt, far away from each terminus. This would rationalize why on average the tryptophans in MSP-1 are the most exposed while the tryptophans in MSP-3 are least exposed.

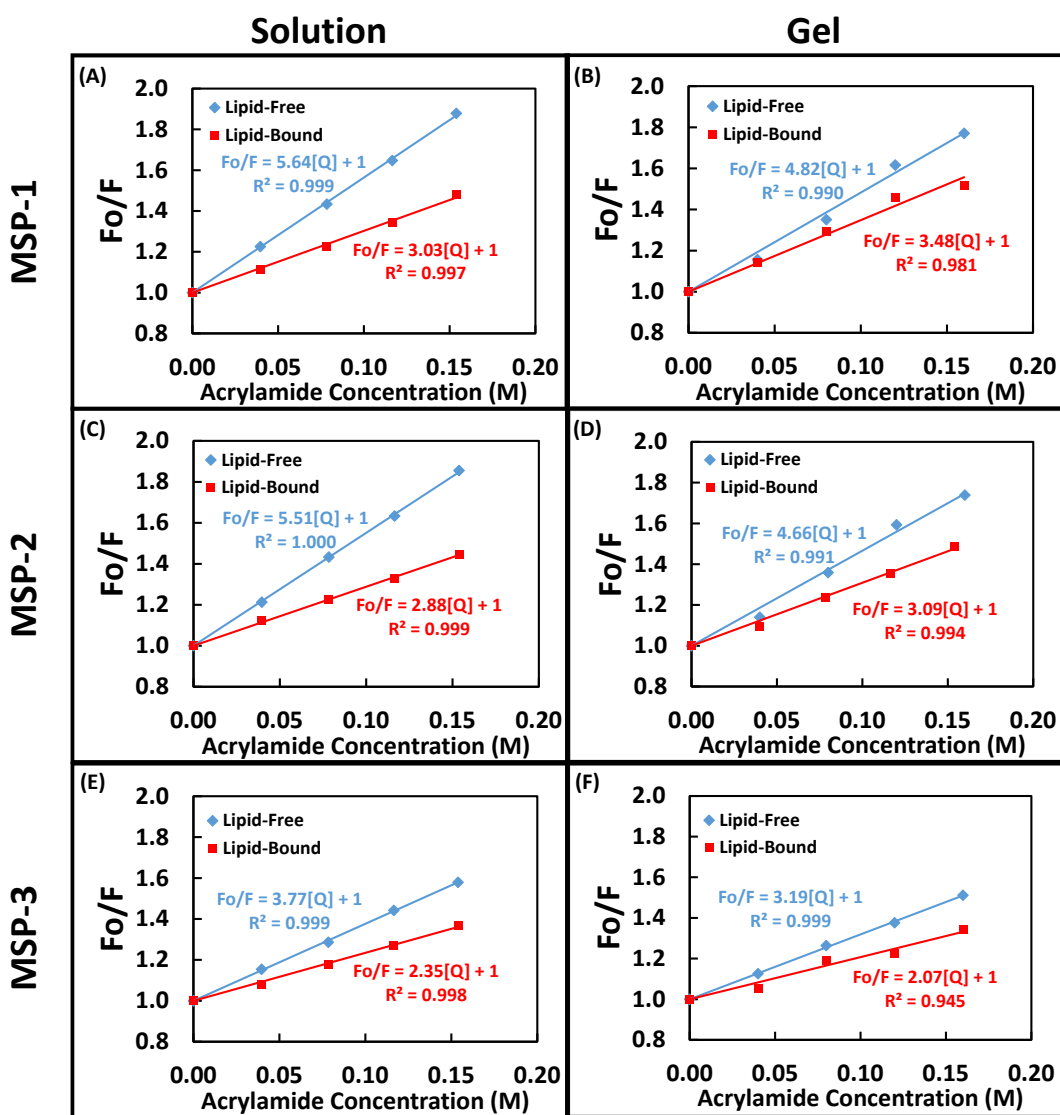


Figure 2: Stern-Volmer plots for lipid free and lipid bound (A, B) MSP-1, (C, D) MSP-2, and (E, F) MSP-3, with corresponding regression Equations. Measurements are for samples (A, C, E) in solution and samples (B, D, F) in silica gel.

Table 2: Stern-Volmer constants ( $K_{SV}$ ) of MSPs in solution and silica gel when quenched by acrylamide

	Solution		Gel	
	Lipid-Free	Lipid-Bound	Lipid-Free	Lipid-Bound
<b>MSP-1</b>	$5.64 \pm 0.10 \text{ M}^{-1}$	$3.03 \pm 0.11 \text{ M}^{-1}$	$4.82 \pm 0.36 \text{ M}^{-1}$	$3.48 \pm 0.34 \text{ M}^{-1}$
<b>MSP-2</b>	$5.51 \pm 0.08 \text{ M}^{-1}$	$2.88 \pm 0.06 \text{ M}^{-1}$	$4.66 \pm 0.33 \text{ M}^{-1}$	$3.09 \pm 0.15 \text{ M}^{-1}$
<b>MSP-3</b>	$3.77 \pm 0.08 \text{ M}^{-1}$	$2.35 \pm 0.06 \text{ M}^{-1}$	$3.19 \pm 0.08 \text{ M}^{-1}$	$2.07 \pm 0.33 \text{ M}^{-1}$

Parallax analysis was used to determine the average position of tryptophan residues of lipid-bound MSPs in solution and in gel with respect to the bilayer center. The helices of membrane scaffold proteins are believed to orient in a manner such that they run perpendicular to the acyl tails of the lipids in the bilayer, as illustrated in Figure 1.<sup>34-35</sup> Therefore, placement of nitroxide quenchers at different heights in the bilayer results in different quenching efficiencies (i.e. quenchers closer to helices quench tryptophan more so than quenchers farther away). Two forms of lipid partitioning stearic acid were used to achieve this effect. One form had nitroxide covalently attached to the 5-carbon (5-DOXYL), while the other had nitroxide covalently attached to the 16-carbon (16-DOXYL). It is expected that 5-DOXYL and 16-DOXYL would align with their acyl tails parallel to the acyl tails of the lipids in NLPs. The chemical structures for both of these quenchers are depicted in Figure 3. The ratio of quenching from nitroxides at these different heights can be correlated to the effective height of tryptophan residues,  $Z_{\text{eff}}$ , using Equation 2. The quenching of tryptophan by 5-DOXYL and 16-DOXYL are shown in Figure 4. For each MSP in both solution and gel, the tryptophan residues were more quenched by 16-DOXYL than 5-DOXYL. This indicates that on average, the tryptophan residues in each MSP were positioned such that they were closer to the nitroxide spin label on 16-DOXYL (i.e. closer

to the bilayer center). Though there is a distinct increase in fluorescence intensities upon gel entrapment for both 5-DOXYL and 16-DOXYL, there is only a slight increase in the effective height from the bilayer center ( $Z_{\text{eff}}$ ) for all MSPs as shown in Table 3. The length of a hydrophobic leaflet on a bilayer is on the order of 15 Å.<sup>46-47</sup> The changes in  $Z_{\text{eff}}$  upon gel entrapment for MSP-1, MSP-2, and MSP-3 are 0.35 Å, 0.14 Å, and 0.21 Å, respectively. Overall, these displacements are very small in comparison to the size of the leaflet (< 3%). All of the values for  $Z_{\text{eff}}$  in Table 3 are consistent with previous works where it has been shown that  $Z_{\text{eff}}$  for tryptophan residues can vary from 4.0 – 6.0 Å depending on the tryptophan location in the scaffold protein.<sup>34-35</sup> This indicates that the MSPs are maintaining a proper lipid-bound conformation upon after entrapment in silica gel due to the minimal change in positioning of the tryptophan residues.

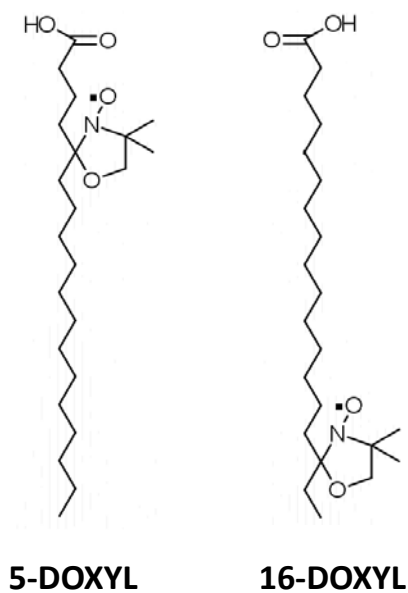


Figure 3: Illustration of the chemical structures of 5-DOXYL and 16-DOXYL.

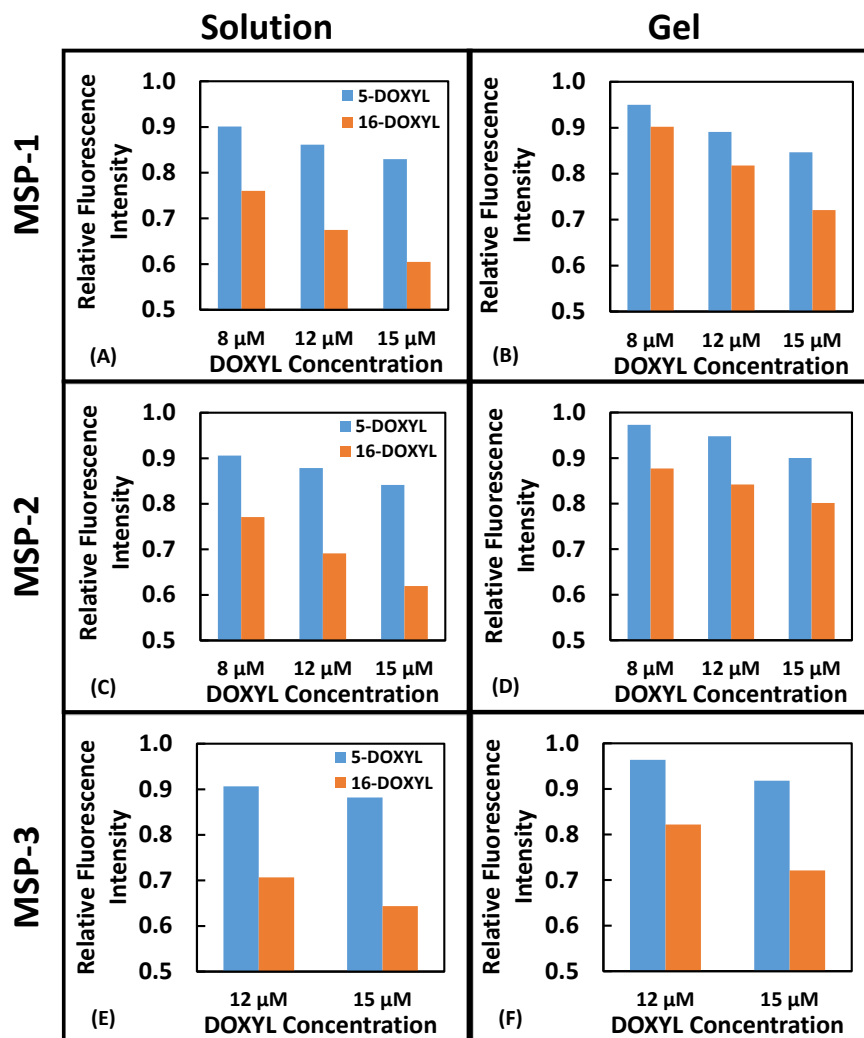


Figure 4: Relative fluorescence intensity of tryptophans in lipid-bound (A, B) MSP-1, (C, D) MSP-2, and (E, F) MSP-3 when quenched by 5-DOXYL and 16-DOXYL at various concentrations. Measurements are for samples (A, C, E) in solution and samples (B, D, F) in silica gel.

Table 3: Effective height ( $Z_{\text{eff}}$ ) of Tryptophan residues in lipid-bound MSPs determined by parallax analysis.

	Solution	Gel
<b>MSP-1</b>	$5.73 \pm 0.01 \text{ \AA}$	$6.08 \pm 0.05 \text{ \AA}$
<b>MSP-2</b>	$5.69 \pm 0.03 \text{ \AA}$	$5.83 \pm 0.19 \text{ \AA}$
<b>MSP-3</b>	$4.92 \pm 0.05 \text{ \AA}$	$5.13 \pm 0.03 \text{ \AA}$

The fluorescence anisotropy of tryptophan in lipid-free and lipid-bound MSPs in both solution and silica gel was analyzed by Equation 3. Fluorescence anisotropy is a measure of rotational freedom of a molecule, which is directly related to its size or the size of the aggregate it is associated with.<sup>38</sup> In Figure 5, it is shown that the anisotropy of each lipid-free MSP is higher than its corresponding lipid-bound MSP in both solution and gel. This difference in anisotropy was determined to be statistically significant (Supporting Information). This indicates that the lipid-free assemblies are larger than the lipid-bound assemblies, due to their slower rotational speed. This is counterintuitive, as one would expect a single protein to be smaller than a protein-lipid assembly consisting of two proteins and a few hundred lipids. However in solution, MSPs exist as oligomers;<sup>30, 41</sup> these oligomers are capable of being larger than NLPs. Using dynamic light scattering, we have confirmed that this is the case, as lipid-free MSPs display a heterogeneous size distribution with many populations larger than that of the homogeneously dispersed lipid-bound population (Supporting Information). There is a decrease in anisotropy for all MSP samples once entrapped in silica gel as shown in Figure 5. This change in anisotropy was also determined to be statistically significant (Supporting Information). Again, this decrease in anisotropy is counterintuitive due to the apparent increased motion in a confined environment. We speculate that this is the result of large lipid-free MSP oligomers breaking down into smaller ones, and NLPs remodeling upon entrapment into smaller particles. The magnitude of the size decrease cannot be determined from our experimental methods. However, our previous work in which the lipid phase behavior of silica-gel entrapped NLPs belted by MSP-3 was investigated corroborate the theory of NLP shrinkage upon entrapment.<sup>27</sup>

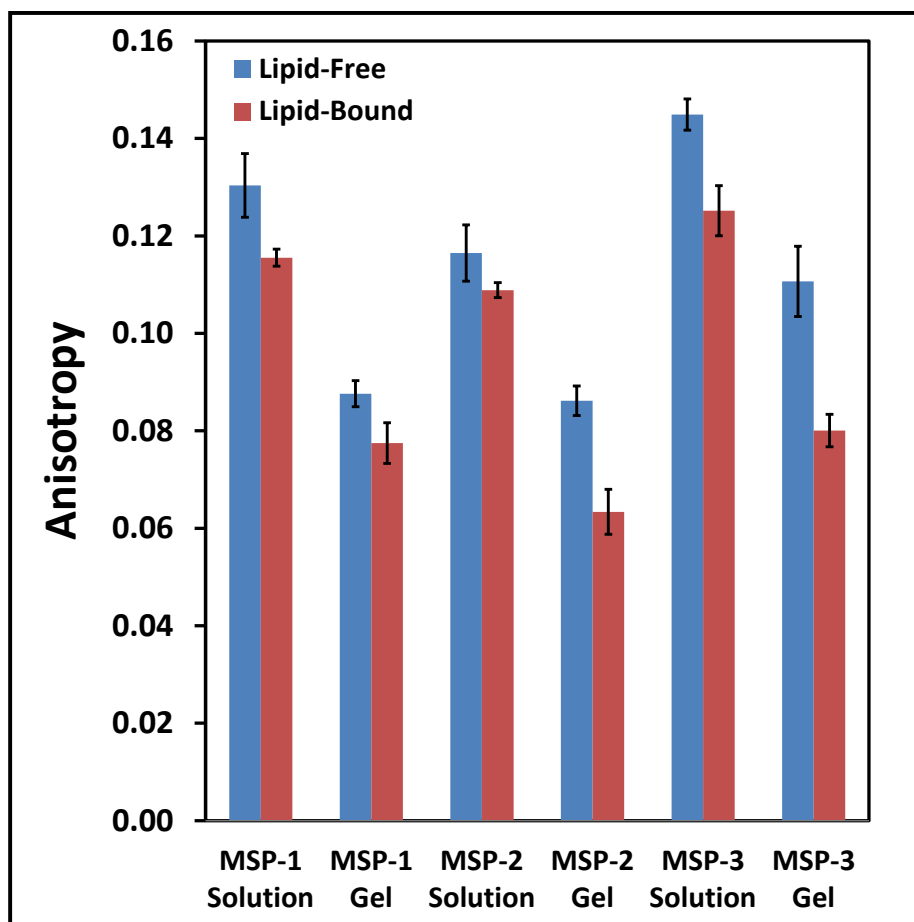


Figure 5: Fluorescence anisotropy values of lipid-free and lipid-bound MSPs in solution and silica gel.

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of MSP-1 and MSP-2 and how they changed upon entrapment. Figure 6 shows the spectra of lipid-free and lipid-bound MSP-1 and MSP-2 in solution and in silica gel. Spectra was not recorded for MSP-3 as we have previously investigated and reported this.<sup>27</sup> All spectra show a curve with two minima at 208 nm and 222 nm, which is indicative of strong alpha-helical content.<sup>48</sup> The corresponding alpha-helical content is shown in Table 4. Figure 6A and 6C show that MSP-1 and MSP-2, respectively, underwent an increase in molar ellipticity when bound to lipids. This corresponds to an increase in alpha helical content, which is shown in Table 4 to be 50% - 72%

for MSP-1 and 48% - 70% for MSP-2. This increase in alpha helical content is consistent with previous works in which Apolipoprotein A-I and other MSPs were examined.<sup>27, 30, 42, 49-50</sup> A similar lipid-induced shift in molar ellipticity of the scaffold protein occurred for NLPs entrapped in the silica gel (Figure 6B and 6D). Thus the results from CD analyses also indicate the MSP maintains its interactions with the lipids when embedded in the mesoporous sol-gel matrix.

Interestingly, silica gel entrapment increased the alpha helical content of lipid-free MSP-1 and MSP-2 (50% - 60% for MSP-1 and 48% - 58% for MSP-2). However, the lipid-bound forms remain almost the same (72% - 70% for MSP-1 and 70% - 72% for MSP-2). This shows that the lipid-free MSPs adopt a more helical structure in the gel, while the lipid-bound MSPs remain relatively unchanged. This is consistent with our previous CD spectroscopy work using MSP-3, where gel entrapment seemed to increase the alpha-helical content of lipid-free MSP-3, but not for the lipid-bound form significantly.<sup>27</sup> This is also corroborated by the acrylamide quenching experiments performed in this work, where the significant decrease in  $K_{SV}$  for lipid-free MSPs suggests a more compact structure in the silica gel. On the other hand, the lack of a significant change in  $K_{SV}$  for lipid-bound MSPs is consistent with the maintenance of alpha-helical content following gel entrapment. Although the speculated size change of NLPs appears to have no effect on lipid-bound MSP structure, perhaps the breaking down of larger lipid-free MSP aggregates into smaller ones permits the formation of structures with higher alpha-helical content, as suggested by the fluorescence anisotropy and circular dichroism measurements. Molecular crowding (volume exclusion) effects of gel entrapment may also drive an increase in the alpha helical content of the lipid-free protein.<sup>51</sup> It is worth noting that in Figure 6, both MSP-1 and MSP-2 seem to exhibit inversion in their peak heights at 208 nm and 222 nm after

silica gel entrapment. Table 5 shows the 222/208 ratio for each sample before and after entrapment. All solution samples have a 222/208 ratio under 1, which is indicative of isolated helices.<sup>52</sup> All of the gel samples have a 222/208 ratio above 1. Though 222/208 ratios above 1 typically indicate the presence of a coiled-coil structures,<sup>52</sup> a coiled-coil motif, would not be justified in the context of our results. For example, a coiled-coil would require the scaffold proteins on an NLP coming into very close proximity of one another. However, the parallax analysis shows that they actually go slightly farther away from one another. Interparticle protein interactions could result in coiled-coils. However, this would indicate that the particles aggregate, thus on average become larger. The fluorescence anisotropy of entrapped NLPs showed that this is not the case, as they may potentially decrease in size after entrapment. We speculate that this inversion in the 222/208 ratio could be a result of interactions of MSP-1 and MSP-2 with the silica gel. The extent and type of interactions involved cannot be determined from this current work and would require further investigation. In our previous work, MSP-3 was utilized as a scaffold protein and also seemed exhibit a 222/208 ratio inversion after gel entrapment.<sup>27</sup> However, this inversion was of a smaller magnitude. In solution, 222/208 ratios were 0.87 and 0.91 for lipid-free and lipid-bound samples, respectively. After entrapment in silica gel, both samples exhibited a 222/208 ratio of approximately 1.0. Based on our analysis, it is difficult to resolve the reasoning for difference in the CD spectral behavior between MSP-1/2 and MSP-3. It could perhaps be related to the size difference between MSP-1/2 and MSP-3. This 222/208 ratio inversion behavior, however, should not detract from the main message of our CD spectroscopy findings; MSP-1 and MSP-2 both exhibit increased alpha-helical content when going from a lipid-free to lipid-bound conformation in solution, and this behavior is consistently observed in gelled samples.



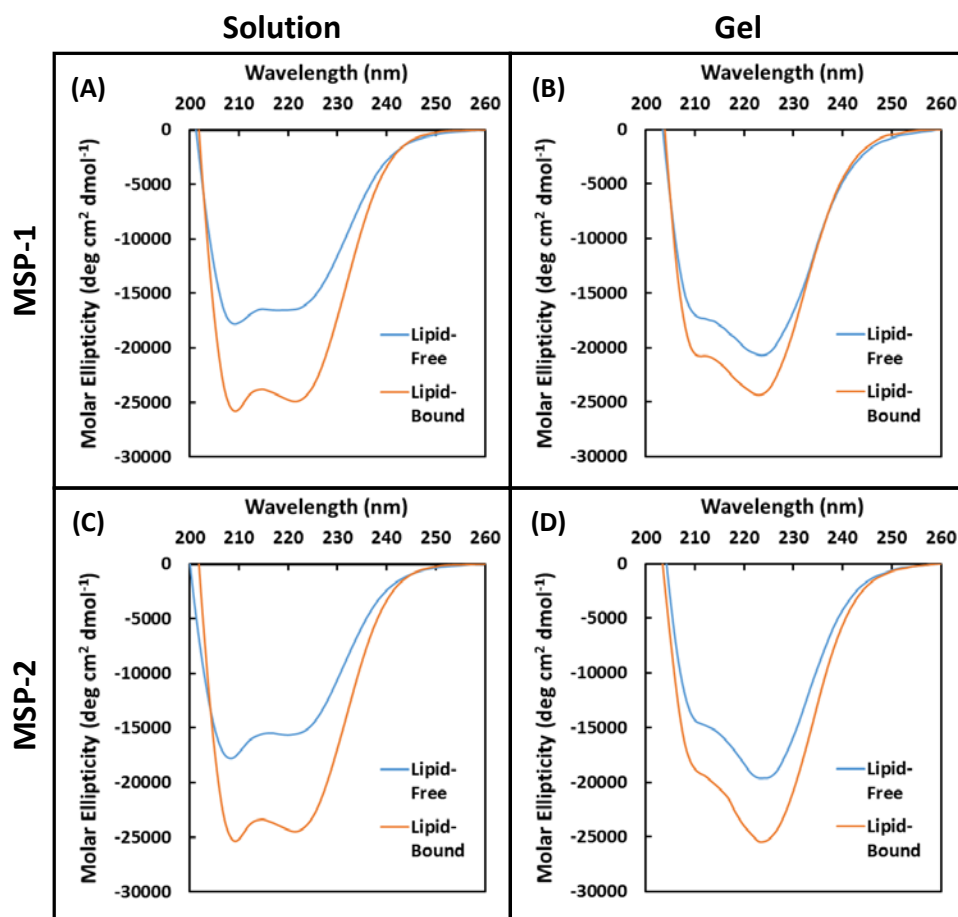


Figure 6: Circular Dichroism spectra of lipid-free and lipid-bound (A) MSP-1 in solution, (B) MSP-1 in silica gel, (C) MSP-2 in solution, (D) MSP-2 in silica gel.

Table 4:  $\alpha$ -helical content of MSPs determined from 222 nm molar ellipticity in circular dichroism spectra

	Lipid-Free	Lipid Bound
MSP-1 Solution	50%	72%
MSP-1 Gel	60%	70%
MSP-2 Solution	48%	70%
MSP-2 Gel	58%	72%

Table 5: 222/208 peak ratio from circular dichroism spectra for MSPs in solution and gel

	Lipid-Free	Lipid Bound
MSP-1 Solution	0.94	0.99
MSP-1 Gel	1.35	1.34

<b>MSP-2 Solution</b>	0.87	0.99
<b>MSP-2 Gel</b>	1.61	1.51

In previous work, we demonstrated that NLPs made using MSP-3 exhibited stability after silica gel entrapment for at least 5-6 weeks.<sup>27</sup> Long term stability of the NLPs examined in this work was not studied. However, based on the general similarities in behavior observed between the three MSPs in this work, we speculate that NLPs synthesized with MSP-1 and MSP-2 are capable of exhibiting time scales of stability similar to those observed with MSP-3. Further analysis would need to be performed in order to assess this prediction.

## **CONCLUSIONS**

We have shown that for three different membrane scaffold proteins (MSPs) associated with nanolipoprotein particles (NLPs), the protein-lipid interactions were retained upon entrapment in TMOS-derived silica gel. The MSPs examined maintained an orientation in which they were buried in hydrophobic lipid tails. This was determined by using fluorescence spectroscopy and fluorescence quenching of tryptophan with acrylamide. Fluorescence spectroscopy showed consistent blue-shifts in tryptophan emission when comparing lipid-free MSP spectra to lipid-bound MSP spectra, which is consistent with MSPs being in a more hydrophobic environment. Fluorescence quenching of the tryptophan residues with acrylamide showed that lipid-bound MSPs had an orientation in which they were less exposed to solvent than lipid-free MSPs. Thus again, they were most likely buried in the lipid tails. Fluorescence quenching using 5-DOXYL-stearic acid and 16-DOXYL-stearic acid showed that lipid-bound MSPs were positioned at a distance of 5-6 Å away from the bilayer center in solution, and maintained that distance of separation after entrapment. Fluorescence anisotropy revealed that NLPs were smaller in size than lipid-free MSP oligomers in both solution and gel, and that both experienced some degree

of shrinkage upon entrapment possible due to structural reorganization. Circular dichroism spectroscopy demonstrated that lipid-bound MSPs possessed a higher degree of  $\alpha$ -helical content in solution than lipid-free MSPs (~70% for lipid-bound and ~50% for lipid-free), and that this higher degree was retained after entrapment (~70% for lipid-bound and ~60% for lipid-free). A higher percentage of  $\alpha$ -helical content is associated with protein-lipid interactions in NLPs. The combination of various fluorescence spectroscopic techniques and circular dichroism spectroscopy illustrated that after entrapment, lipid-bound MSPs exhibited much less significant structural and dynamic changes than lipid-free MSPs due to stabilization from the protein-lipid interactions. Our spectroscopic analyses confirmed that MSPs indeed continued to interact with lipids in NLPs after entrapment with minimal change. In combination with our previous work, we have demonstrated that NLPs are viable biological membrane hosts for silica gel entrapment and more suitable than liposomes. This sets precedent for future work utilizing integral membrane proteins incorporated into NLPs to synthesize functional biomaterials derived from sol-gel encapsulation.

## **SUPPORTING INFORMATION**

The Supporting Information provides additional details of fluorescence emission spectral analysis methods and statistical analysis, including all regressed spectral data from the quenching of tryptophan fluorescence by acrylamide. It also provides N<sub>2</sub> adsorption isotherm data used to determine silica gel pore size and graphical dynamic light scattering data used to characterize MSP aggregates and NLP diameters. This material is available free of charge on the internet at <http://pubs.acs.org>.

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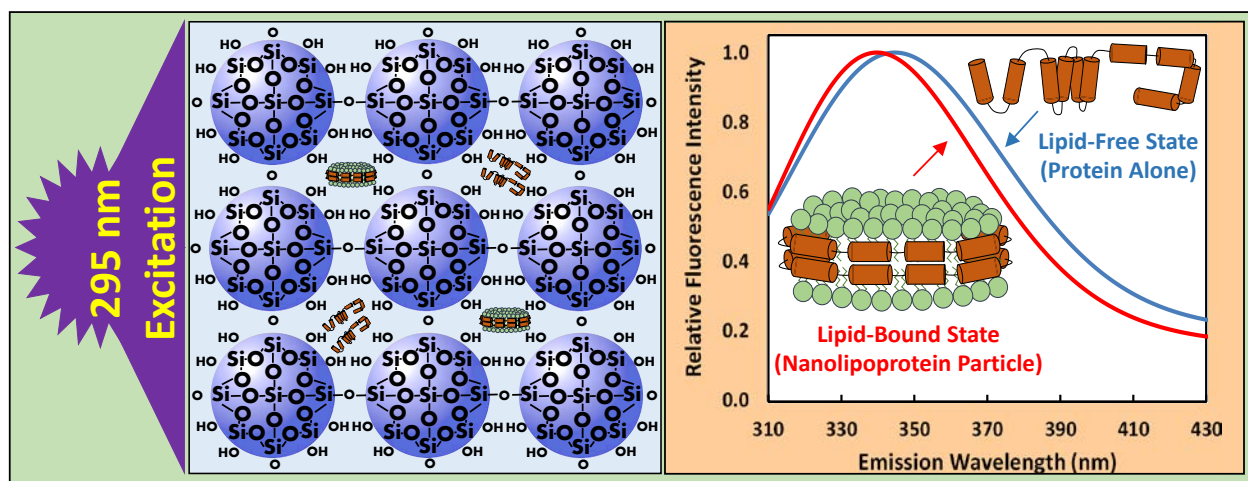


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Abstract Graphic