

# Immobilization in Surface-Tethered Lipid Vesicles as a New Tool for Single Biomolecule Spectroscopy

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Single-molecule fluorescence studies of functional biomolecule dynamics rely on the ability to provide biologically relevant experimental conditions. Long measurement times on single molecules require their immobilization, which might modify their dynamics through interactions with the trapping medium, e.g., a glass surface or a polymer gel. In an effort to overcome this problem we have devised a new immobilization technique, based on the confinement of single biomolecules inside 100 nm surface-tethered lipid vesicles. The number of molecules in each vesicle can be accurately determined from fluorescence time traces; under our experimental conditions the number distribution of encapsulated molecules obeys a Poisson distribution with an average occupancy of 0.65 molecules per vesicle. It is further shown that the distribution of fluorescence polarization values of trapped molecules can serve as a sensitive probe for their freedom of motion and thus for the environment they sample inside the liposomes. Polarization distributions are obtained for two vesicle-entrapped labeled proteins, bovine serum albumin and adenylate kinase, and compared with distributions measured for the same proteins directly adsorbed on glass. From the significant relative narrowing of the distributions for encapsulated molecules, it is concluded that their motion within the vesicles is quite similar to free solution.

## Introduction

Single molecule fluorescence microscopy has evolved into a powerful technique for the elucidation of functional dynamics of biomolecules. (For reviews see refs 1–4.) One of the main fortes of this technique lies in its ability to follow the temporal trajectory of a single functional biological molecule for a long time and unmask dynamic events, which are completely hidden in ensemble measurements.<sup>5–9</sup> The success of such measurements relies on the availability of experimental conditions, which are directly relevant to the biological function under study. One possible way to achieve this is to perform measurements on individual protein molecules freely diffusing in a buffer solution. This can be done using confocal microscopy techniques, where bursts of photons are collected from a small volume illuminated by a tightly focused laser beam, as a molecule traverses this volume.<sup>10,11</sup> Such measurements (sometimes termed “burst spectroscopy”), while providing the most native-like environment for water-soluble biomolecules, are limited by the short residence time of each molecule within the illuminated volume, dictated in turn by its diffusion constant. Extended observation periods of individual molecules are important for the identification of slow or rare dynamic events. To enable observation periods much longer than allowed by diffusion, it is necessary to isolate and immobilize molecules. Several immobilization methods have been reported in the literature. For example, biomolecules were adsorbed nonspecifically to a surface.<sup>12,13</sup> Molecules were also specifically attached by using charge interactions,<sup>14</sup> coordination of a histidine tag to nickel complexes,<sup>15</sup> or binding of a biotin moiety to an avidinylated surface.<sup>16,17</sup> Entrapment in the pores of poly(acrylamide)<sup>6</sup> or agarose<sup>7</sup> gels was also found to be useful for single molecule immobilization. All of these methods lead to a strong interaction

of the molecule with its immediate environment, possibly modifying its energy landscape. Such effects might be especially deleterious for studies of protein folding on the single molecule level. A denatured protein might expose hydrophobic residues which will tend to interact with the surface through its long-range van der Waals potential. Surface electrostatics<sup>18</sup> might also modify the properties of the fluctuating protein. As for gel entrapment, it might create steric hindrance and restrict the conformational space available to the folding protein. Surface effects were directly exposed in the recent protein folding study of Hochstrasser and co-workers,<sup>19</sup> where it was shown that the end-to-end distance distribution of surface-tethered denatured polypeptides is much broader than the distribution obtained under similar conditions in free solution.

Here we report a novel immobilization technique for biomolecules, which allows us to overcome the problem of molecule–surface interaction. We trap single protein molecules inside lipid vesicles,<sup>20</sup> which are in turn tethered to a supported bilayer on glass via biotin–avidin chemistry. We present evidence that the vesicles remain intact after attaching them to the surface. We also present measurements of the fluorescence from single vesicles, and show that we are able to directly determine the number of fluorescent molecules encapsulated in each. Using polarization spectroscopy, which has been proven to be a valuable technique for studying individual molecular orientation,<sup>12,21–33</sup> we confirm that the motional freedom of trapped protein molecules is similar to that in free solution. Vesicle encapsulation is thus shown to be a promising technique for studies of single-molecule large-scale protein dynamics.

## Methodology

To prepare large unilamellar vesicles (LUV) encapsulating protein or dye molecules, we used the following procedure. A quantity of egg-phosphatidylcholine (PC), mixed with 0.2% of biotinylated phosphatidylethanolamine (PE) in *tert*-butyl alcohol,

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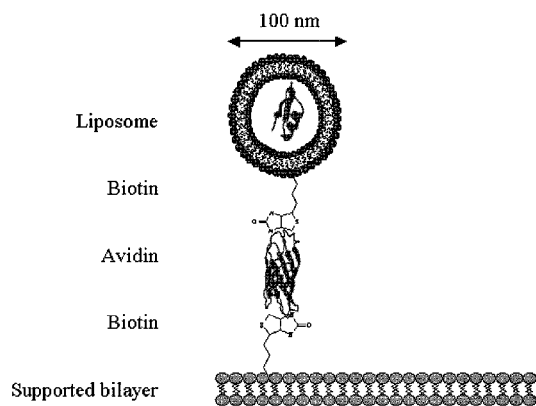
was first lyophilized and then rehydrated by a buffer solution, thus creating multilamellar vesicles. Molecules to be encapsulated were added to the solution, which was then extruded repetitively through a polycarbonate film with 100 nm pores.<sup>34</sup> This procedure produced LUV with a fairly narrow size distribution peaking at the pore size, as determined by dynamic light scattering. A protein concentration of  $\sim 3 \mu\text{M}$  was used in order to achieve an average protein/vesicle ratio of 1:1, assuming perfect trapping efficiency.

Loaded vesicles were tethered to a glass surface covered with a biotinylated supported lipid bilayer. This bilayer was formed by adding a solution containing unloaded PC/0.2% biotin-PE liposomes to a cell made of two hydrogen fluoride etched glass slides separated by Parafilm spacers and rinsing the cell after a short incubation period. It is well known that liposomes will fuse and spread on a glass surface to form a lipid bilayer.<sup>35,36</sup> We verified that this indeed occurred under our conditions by using fluorescently labeled lipids. Imaging of the obtained supported bilayer showed a homogeneous surface coverage. In a second step we bound avidin to the biotinylated membrane by adding a solution containing 1 mg/mL avidin into the cell. Finally, protein- or dye-loaded biotinylated liposomes were added and allowed to interact with the surface-adsorbed avidin.

At a vesicle concentration of  $3 \times 10^8$  per  $\mu\text{L}$  (total lipid concentration of 0.01%), attachment was found to be essentially specific, i.e., biotin-avidin mediated. In fact, in the absence of avidin there was an order of magnitude reduction in the number of vesicles attached to the surface. We note that nonspecifically bound vesicles are likely to leak their contents by fusing with the surface bilayer. However, if they do remain stable they are identical, for our purposes, to specifically bound ones. By gauging the incubation time for vesicle attachment we were able to achieve low enough surface coverage to allow single vesicle-single molecule detection. The concentration of biotinylated lipids used ensured that each vesicle was strongly anchored to the surface through the interaction of several biotin-avidin pairs.

While some fluorescence measurements were carried out on a commercial confocal microscope (Olympus), most of the experiments reported in this paper were conducted with a home-built confocal microscope system. This system is based on an Axiovert 135 TV inverted microscope (Zeiss) equipped with  $100\times$  NA 1.30 (Fluar, Zeiss) objective. The sample was illuminated by the 532 nm beam of a diode-pumped frequency-doubled Nd:YVO laser (Verdi, Coherent), focused through the objective. In some experiments a two-photon excitation scheme was implemented, and the excitation source used was an 80 MHz mode-locked Ti:sapphire laser (assembled from a TS model laser kit, Kapteyn-Murnane laboratories). Samples were scanned on a piezoelectric X-Y scanning stage (Nanonics) controlled by an SPM 100 controller (RHK Technologies). The emitted light was collected by the objective and then passed through either OG-570 filters (Schott) for 532 nm excitation or BG-39 filters (Schott) for two-photon excitation. Fluorescence was collected with a photon-counting avalanche photodiode (EG&G), typically with a time window of 2 ms. After obtaining an image of a  $7 \mu\text{m} \times 7 \mu\text{m}$  on the sample, the scanning stage was shifted to position a single molecule/vesicle under the laser beam and photons were collected from this molecule until photobleaching was observed.

In fluorescence polarization experiments we excited molecules with circularly polarized light, thereby avoiding photoselection. The vertically and horizontally polarized components of the emitted light,  $I_V$  and  $I_H$ , were split using a polarizing cube and collected with two photon-counting avalanche photodiodes. The



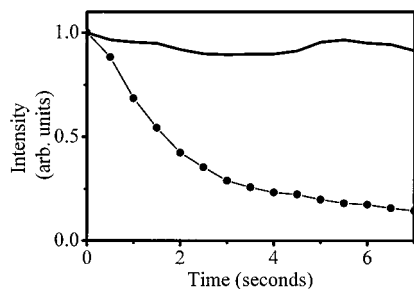
**Figure 1.** Scheme of the methodology used for surface tethering of single molecule-containing liposomes. Large unilamellar lipid vesicles, each encapsulating a single protein molecule, were attached to a glass-supported lipid bilayer, using biotin-avidin chemistry.

fluorescence polarization ( $P = (I_V - I_H)/(I_V + I_H)$ ) was calculated for each data point and then averaged over the whole time trace accumulated for each individual molecule.

## Results and Discussion

A multistep process, based on the familiar biotin-avidin interaction, was used to tether vesicles loaded with protein molecules to a glass surface, as depicted in Figure 1. We first formed on the surface a supported bilayer made of PC with a small amount of biotinylated PE, to which avidin was bound. Biotinylated vesicles loaded with protein or dye molecules were then bound to the avidinylated surface. The size of the vesicles used in this work, 100 nm, was selected to be significantly smaller than the size of a diffraction-limited laser spot in a fluorescence microscope ( $\sim 0.5 \mu\text{m}$  for a 532 nm laser beam and a high numerical aperture objective), yet much larger than the size of a protein molecule, so as to allow ample volume for essentially free molecular motion. The simple chemical composition of the vesicles was chosen so that the lipid bilayer will be essentially neutral, thus minimizing interactions with encapsulated molecules. Surface tethering of the liposomes allows facile data collection from many single molecules, either by sample scanning confocal microscopy (which we demonstrate here) or by large-area imaging techniques suitable for single molecule detection, such as total internal reflection microscopy.<sup>37</sup> Our approach is thus complementary to the work of Zare and co-workers, who studied enzyme-substrate interactions in *single giant vesicles* held in an optical trap.<sup>38,39</sup>

The success of our method relies on the lack of leakage of the contents of tethered liposomes. Several schemes have been proposed to demonstrate the integrity of surface-attached vesicles, including the application of a quartz crystal microbalance,<sup>40</sup> rupturing the vesicles and measuring their contents quantitatively,<sup>41</sup> or measuring dequenching of a fluorescent dye encapsulated at a high concentration.<sup>42</sup> We found it useful to employ an in-situ fluorescence technique, which allowed us to follow over time the integrity of surface-tethered vesicles. Lipid vesicles were loaded with a high concentration of a fluorescent dye, either hydroxypyrene trisulfonate (HPTS) or Cy3 (25 mM and 2.6 mM, respectively). Both of these dyes are negatively charged in the pH 9 buffer we used and are therefore highly soluble and will not adsorb on any of the lipid surfaces in the sample. A solution of the dye-loaded liposomes was added on top of an avidin-covered surface (prepared as described above), and the fluorescence was measured in a fluorescence micro-

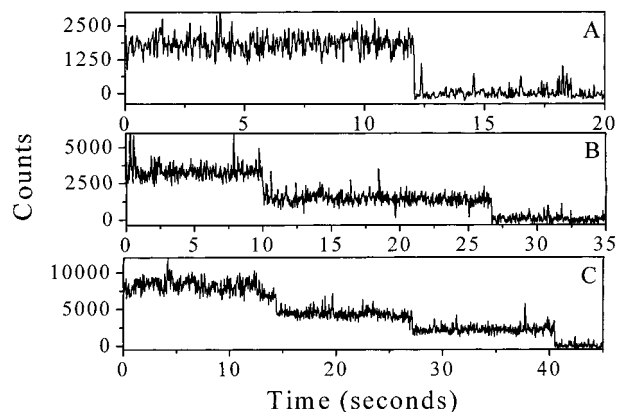


**Figure 2.** Fluorescence of 25 mM of hydroxypyrene trisulfonate in vesicles. In freely diffusing vesicles (—) the fluorescent signal remains constant in time. In surface-bound vesicles (---) the signal decays as the dye photobleaches.

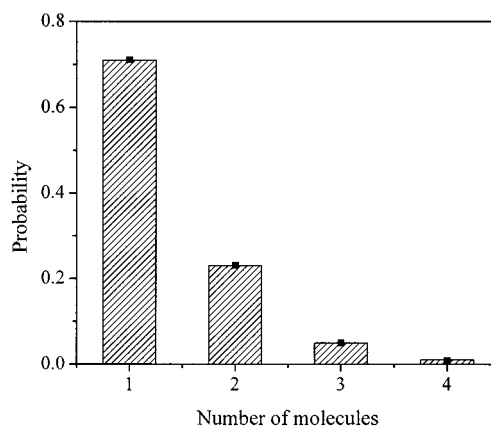
scope, with the excitation laser focused first well inside the solution. It was found that the fluorescence intensity is constant over time and does not show any photobleaching, as expected for a situation where the vast majority of the fluorophore-containing vesicles are freely diffusing. We then rinsed all liposomes that were not adsorbed on the surface and repeated the fluorescence measurement, now focusing the exciting laser beam on the glass surface. We found that in this case the fluorescence intensity decays with the characteristic time scale of the dye photobleaching reaction. Sample data from the HPTS experiment are shown in Figure 2. Similar behavior to that shown in the figure could be reproduced by moving the laser beam to new spots on the sample. Further rinses of the flow cell did not significantly reduce the initial fluorescence intensity, indicating no leakage of vesicle contents. Finally, as a control, we repeated the above steps with unencapsulated dye, in order to discard dye adsorption to either avidin molecules or to the supported bilayer. We found no evidence for such adsorption. Thus it seems that the supported bilayer on the surface prevents fusion of the loaded liposomes with the glass and allows them to remain intact and keep their contents.

Encapsulation of single molecules inside vesicles is expected to be a probabilistic process, governed by Poissonian statistics. Thus one expects that some fraction of the vesicles will contain more than one molecule. The success of the vesicle trapping method relies on our ability to detect these vesicles and discard data collected from them from any further analysis. We found that this can be achieved by a simple procedure carried out during the analysis of fluorescence time traces obtained from single vesicles. Since the photobleaching of each molecule occurs in a single step, the occurrence of more than one molecule showed as a succession of photobleaching steps in the time trace. Sample time traces collected from vesicles with one, two, and three molecules of the protein adenylate kinase (AK), labeled specifically at position 142 with the dye Texas Red, are shown in Figure 3. By counting the number of molecules in more than 300 vesicles we were able to construct the vesicle occupancy probability distribution, which is plotted in Figure 4. The distribution fits very well a calculated Poisson distribution with an average occupancy of 0.65 molecules per vesicle. This number matches nicely the expectation based on the concentration of protein molecules introduced during vesicle preparation and on the encapsulation efficiency. For any further analysis we used either data from vesicles containing a single molecule or truncated data from the last photobleaching step in the case of vesicles with more than one molecule.

Vesicle-encapsulated protein molecules might either reside in the water compartment or interact strongly with the lipid bilayer and even adsorb to it. A molecule diffusing in the water compartment of a vesicle will still often collide with the lipid



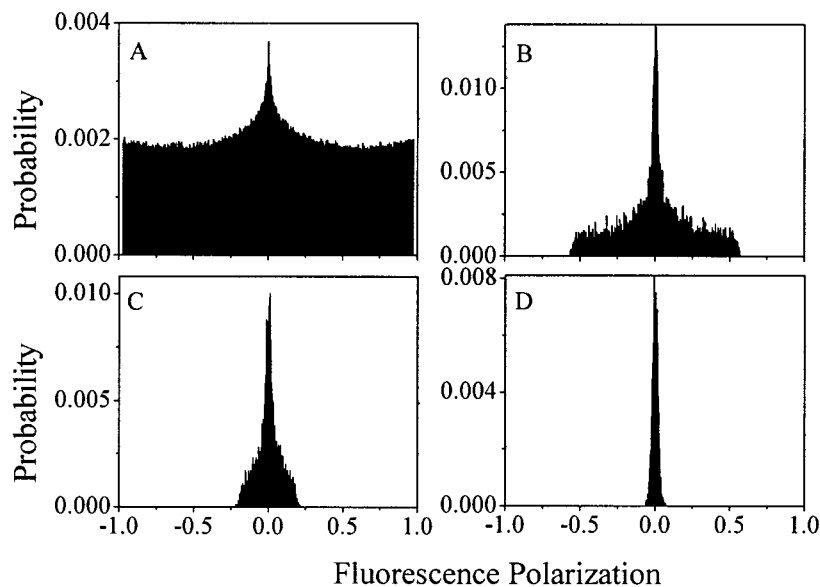
**Figure 3.** Time traces of the fluorescence of individual lipid vesicles encapsulating Texas Red-labeled adenylate kinase molecules. The number of sudden photobleaching steps provides the number of molecules in each vesicle. Panel A shows the time trace of a single molecule in a vesicle, panel B shows the time trace of two molecules, and panel C shows the time trace of three molecules.



**Figure 4.** Vesicle occupancy probability distribution, as calculated from measurements of 323 individual vesicles containing Texas Red-labeled adenylate kinase molecules. The bars show the probability of finding a particular number of molecules in a vesicle. The black squares show the calculated Poisson probability with a mean occupancy of 0.65 molecules per vesicle, which matches nicely the experimental results.

wall.<sup>39</sup> This is quite similar to what happens inside living cells, where protein molecules encounter many membranous compartments, but nevertheless the structure and function of a protein might be affected by this interaction. It is thus important to test where encapsulated molecules reside and which environment they mostly sample inside a liposome. One possible way to answer this question is to study the orientational motion of trapped molecules. We used polarization microscopy to show that protein molecules in vesicles possess rotational freedom similar to molecules diffusing in free solution. The experiment involved a comparison of the distribution of fluorescence polarization values of individual fluorescently labeled protein molecules immobilized directly on a glass surface or encapsulated in surface-tethered vesicles. Before presenting the experimental results we analyze the expected shape of the fluorescence polarization distribution under different conditions.

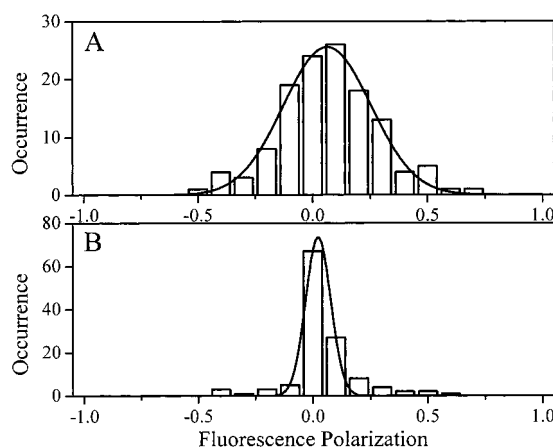
It is expected that the transition dipole moment of an immobile fluorophore will have a distinct and unvarying orientation in space. In an optical collecting system with a numerical aperture approaching zero, the two polarization components,  $I_V$  and  $I_H$ , can be directly related to the direction of the transition dipole moment projection on the image plane. In a high numerical aperture optical system this is not true anymore, as some of the light polarized in the direction



**Figure 5.** Simulations of the distribution of fluorescence polarization values for randomly oriented protein molecules labeled with fluorescent tags with a varying level of motional freedom. It is assumed that the fluorescence is collected by a microscope objective with a numerical aperture of 1.3. (A) No freedom of motion for either protein molecule or fluorescent label. (B) Each fluorescent label is allowed to wobble in a cone with a semiangle of  $50^\circ$ . (C) Each fluorescent label is allowed to wobble in a cone with a semiangle of  $75^\circ$ . (D) Each protein molecule exhibits very fast free rotation on the sampling time scale.

perpendicular to the image plane will also be collected, and will contribute equally to  $I_V$  and  $I_H$ , thus leading to some depolarization of the fluorescence signal.<sup>43</sup> A simulated distribution of fluorescence polarization values for randomly oriented immobile molecules is shown in Figure 5A. For this distribution, 100 000 transition dipole directions were randomly selected and the polarization for each was calculated, assuming fluorescence collection through an objective with a numerical aperture of 1.3. The distribution of Figure 5A is very broad, with a sharp spike at zero polarization, which is due to molecules oriented with their transition dipole moment nearly perpendicular to the image plane. In a real experiment, these molecules will barely be detected over background, due to the low probability of exciting them and detecting photons they emit.<sup>44,25</sup> The sharp peak will therefore be significantly diminished.

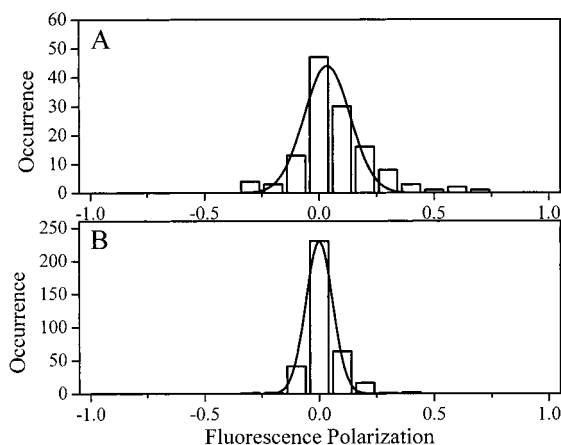
The polarization distribution gets narrowed if the fluorescent molecules possess some limited freedom of motion, as might be expected for a fluorescent tag on the surface of a protein. Figures 5B and C show simulated polarization distributions for the case that the fluorophore is free to wobble in a cone of  $50^\circ$  and  $75^\circ$ , respectively. It is assumed that the wobbling is much faster than the sampling time of the experiment, so that each time the molecule emits a photon the transition dipole moment is randomly reoriented within the cone. Thus each point in the distribution was obtained by randomly selecting a transition dipole direction as above, then calculating the polarization for 100 random orientations within a specified cone around this direction, and finally averaging over these 100 values. This process was repeated 10 000 times to form the final distribution. Note again that the large spike at zero polarization will be diminished in a real experiment. The figures clearly demonstrate the extent of distribution narrowing and its dependence on the wobbling cone semiangle. Besides fluorescent tag wobbling, other factors may also contribute to the narrowing of the polarization distribution, such as partial rotational motion of the protein molecules themselves, or nonrandom orientation of surface adsorbed molecules. In the limit where protein molecules are freely rotating much faster than the sampling time, the direction of the transition dipole moment of each molecule is



**Figure 6.** Distribution of fluorescence polarization values of individual tetramethylrhodamine-labeled bovine serum albumin molecules. (A) 127 molecules directly adsorbed on a glass surface. The broad distribution indicates limited rotational motion. (B) 123 molecules encapsulated in surface-tethered lipid vesicles. The narrow distribution provides evidence for free rotation on the measurement time scale. The solid curves in both panels are Gaussian fits to the experimental data.

fully averaged and the fluorescence polarization distribution is a narrow peak at zero, as shown by the simulation Figure 5D. Overall, the simulations indicate that the width of the fluorescence polarization distribution can indeed teach us about the motional freedom experienced by the probed molecules, in cases where the motion is faster than the time resolution of the experiment.

Measured distributions of fluorescence polarization values of single bovine serum albumin (BSA) molecules labeled with tetramethylrhodamine are presented in Figure 6. The distribution for 127 protein molecules immobilized directly on a glass surface is shown in Figure 6A. This distribution is very broad, and a Gaussian fit gives a full width at half-maximum (fwhm) of 0.45. It is significantly narrower though than the distribution expected for completely immobile molecules, which is probably due to the local motion of the fluorescent labels, as suggested by the simulations above. In Figure 6B we show the distribution



**Figure 7.** Distribution of fluorescence polarization values of individual Texas Red-labeled adenylate kinase molecules. (A) 128 molecules directly adsorbed on a glass surface. The broad distribution indicates limited rotational motion. (B) 356 molecules encapsulated in surface-tethered lipid vesicles. The narrow distribution provides evidence for free rotation on the measurement time scale. The solid curves in both panels are Gaussian fits to the experimental data.

of fluorescence polarization for 123 BSA molecules trapped inside surface-tethered liposomes. The very narrow, zero-peaking distribution (fwhm = 0.13 from the fit) indicates freedom of rotational motion inside the vesicles.

As a second example, we present in Figure 7 distributions of fluorescence polarization values of single Texas Red-labeled AK molecules. The distribution for 128 protein molecules immobilized directly on a glass surface is shown in Figure 7A. This distribution is narrower than in the previous case (fwhm = 0.24), which probably indicates a larger local rotational freedom for the Texas Red label. This is not unexpected, as this label is attached to the protein through a linker that is longer than that of tetramethylrhodamine. Nevertheless, the distribution obtained for AK molecules trapped in vesicles, which is shown in Figure 7B, is significantly narrower (fwhm = 0.14) and matches again the expectation for freely rotating molecules.

The data on both BSA and AK indicate that encapsulated protein molecules reside mainly in the water compartment of the vesicles, as expected for water-soluble globular proteins. If the molecules were to adsorb on the lipid membrane, we would expect the polarization distributions to be more similar to those of glass-adsorbed molecules. This is because the motions of the large and strongly surface-tethered vesicles are not expected to contribute to the overall motion of the molecules within the sampling time of the experiment, 2 ms. In fact, the rotational motion of a free 100 nm vesicle should be on the hundreds of microseconds time scale, and multiple-point tethering (each vesicle is tied to the surface by several avidin–biotin pairs) restricts it even further. A degree of motional freedom similar to that seen here was inferred from polarization distributions by Brasselet et al.<sup>45</sup> for single protein molecules in a gel, albeit with a sampling time of 20 ms.

We further note that no evidence for rotational motion on a time scale *slower* than the 2 ms sampling time was detected in our experiments, either for glass-adsorbed or vesicle-encapsulated molecules, apart from a few polarization jumps that occurred in a very small number of molecules. This is different from the results of Weiss and co-workers with the enzyme staphylococcal nuclease on a surface, where slow reorientational motion with an average correlation time of  $\sim 200$  ms was detected.<sup>46</sup> It is possible that the phenomenon detected by these workers is sensitive to the specific protein under study, to its

particular mode of surface adsorption, or to the position of attachment of the fluorescent probe to it. We cannot completely overrule some slowing down of rotational motion of encapsulated proteins compared to freely diffusing proteins, since the free-solution rotational correlation time of these molecules is much shorter than the sampling time (for BSA, for example, this time is  $\sim 50$  ns<sup>47</sup>). In principle, it might be possible to increase the time resolution of our experiment by a factor of 10–100, by increasing the laser excitation power until saturation of the fluorophore transition<sup>48</sup> (the price is, of course, a shorter total observation time on each molecule before photobleaching). Fluorescence anisotropy decay measurements on single molecules, using time-correlated single photon counting techniques, might also help shed more light on the motion of molecules inside vesicles, although the fluorescence lifetime of many labels is too short to accurately probe overall rotational motion. With the above considerations in mind, our experiments do provide clear evidence that vesicle-encapsulated proteins sample environments more similar to free solution than do adsorbed proteins.

## Conclusion

We have shown that it is possible to tether intact lipid vesicles to a glass surface using a simple stepwise process and to select those vesicles that trap a single molecule. Further, the vesicles provide a native-like environment for encapsulated proteins, as evidenced by unhindered rotational diffusion of the trapped molecules. The new tool developed here is expected to be especially valuable for studies of large conformational changes of individual soluble proteins, as well as for studies of protein folding/unfolding on the single molecule level. Vesicle entrapment should prove useful even for single molecule studies using burst spectroscopy, where it will also allow extended observation times for each molecule, due to the slower diffusion times of vesicles. One apparent caveat of the liposome technique is that it does not allow facile buffer exchange during the experiment, which might be important for triggering a functional transition in a biomolecule. Methods to overcome this problem might be developed, based on the use of membrane permeating agents, or on controlled photorelease of chemical modifiers co-trapped within the vesicles. It should be stressed, though, that much information can be gleaned in equilibrium measurements of protein dynamics, where the focus is on analysis of fluctuations around a thermodynamically fixed point.<sup>7,19</sup> Such analysis is the centerpiece of the now-routine single channel voltage recording techniques.<sup>49</sup> Finally, surface-tethered vesicles can be used for single molecule experiments on reconstituted membrane proteins and peptides. While supported bilayers have proved to be useful tools for studying single molecules freely diffusing and reorienting in the membrane plane,<sup>30</sup> the investigation of functional dynamics in membranes might require immobilization, naturally provided by liposomes.

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