Formation of Giant Unilamellar Vesicles from Block Copolymers and Study of AqpZ and KvAP Protein Incorporation with Light Microscopy

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ABSTRACT

We have formed giant unilamellar vesicles (GUV's) using two types of block copolymers—PMOXZ-PDMS-PMOXZ triblock and PBD-PEO diblock—and studied incorporation of labeled, transmembrane proteins using aquaporin (AqpZ), a water channel protein, and KvAP, a voltage-gated potassium channel. To form GUV's, we adapted several techniques originally used to form giant lipid vesicles, including sonication, detergent-removal, and film hydration. In different experiments, proteins were labeled with fluorescent dyes and gold nano-particles. Vesicle formation and protein insertion was observed using DIC and fluorescence microscopy. GUV's ranging from 1-50 um formed with each of the polymers. There was a strong tendency for the PMOXZ-PDMS-PMOXZ triblock to form multilamellar vesicles, while the film hydration technique consistently produced GUV's using the PBD-PEO diblock.

Keywords: polymer vesicle, protein incorporation, GUV, AqpZ, KvAP

1 INTRODUCTION

The term giant vesicles can include all vesicles visible with the aid of an optical microscope. Their size permits the use of many techniques originally developed for cells, such as micromanipulation and microinjection. The theory linking amphiphiles to vesicle formation is still not well understood, and many molecules do not organize to produce giant vesicles [1, 2]. Empirical studies observing vesicle formation therefore provide critical information.

Although block polymer molecules assembled into a membrane may be organized similarly as lipids, they are individually much more massive molecules with unique elements. Still, researchers have successfully formed giant vesicles using certain block copolymers. In particular, Meier et al. have reported giant vesicles formed using a polymethyloxazoline-polydimethylsulfoxide-polymethyloxazoline (PMOXA-PDMS-PMOXA) triblock polymer [3, 4]. Likewise, working with polybutadiene-polyethyleneoxide (PBD-PEO) diblock copolymers, Discher et al. have formed giant vesicles and conducted studies of mechanical properties [5, 6]. This work expands on these studies, evaluating various methods of giant vesicle formation and protein incorporation with similar block copolymers.

Methods where vesicles form in the absence of solvent are generally a prerequisite for functional protein. In thin film rehydration, the polymer is first dissolved in a solvent, applied to a surface, and dried under vacuum. Buffer is added to the surface to initiate vesicle formation [2, 3, 7, 8]. Preparation on glass slides allows vesicle swelling to be observed using a microscope. Because vesicles form attached to a surface, they are easy to locate and micromanipulate.

A different technique which we explore in these experiments is the preparation of giant vesicles through sonication, a variation on a method used to produce small lipid vesicles (<30 nm). It is very rapid, solvent-free, and yields predominantly multi-lamellar vesicles. This procedure can perhaps be described as bulk swelling with an extremely high amount of agitation. The mechanism of vesicle formation is possibly a greatly accelerated swelling process.

For these experiments, both KvAP and AqpZ were modified with polyhistidine-tags for purification through Ni-NTA affinity chromatography, which also allows the use of a number of labeling techniques targeting the tags. KvAP and AqpZ also have cysteine residues near the ends of the proteins, allowing thiol labels to easily be applied. Nanogold and quantum dot antibody conjugates can specifically target the polyhistidine sequences present on both proteins. Anti-His Nanogold in conjunction with TEM has been previously used to detect protein incorporation in submicron ABC triblock polymers [9].

Reconstitution is relatively straightforward with lipid—detergent solubilized membrane proteins are added to partially solubilized liposomes and detergent removed [10]. With film rehydration, a film can be partially dried from smaller proteo-vesicles and rehydrated to yield giant vesicles with incorporated protein [11, 12].

Protein incorporation in polymer giant unilamellar vesicles (GUV's) could allow detailed measurement of protein function in unusual membranes. For example, KvAP, a voltage-gated potassium channel similar to those found within neurons, may behave radically different within a thicker or less fluid membrane. A patch-clamp study could provide information about how the membrane influences protein gating and conduction, useful for electrophysiology, and a prerequisite to creating complex hybrid protein-polymer devices.

2 MATERIALS AND METHODS

Polymers were purchased from Polymer Source, Inc. and based on published results of Meier et al. and Lee et al.[5, 13]. Two versions of the $PMOXZ_m$-$PDMS_n$-$PMOXZ_m$ polymer were used—M_n=2.0-4.0-2.0x10^3 (long) and M_n=1.1-2.5-1.1x10^3 (short). Diblock PBD-PEO M_n=2.5-1.3x10^3 KvAP and AqpZ were purified through NTA affinity in protocols modified from Ren et al. [14] and Kozono et al.[15] respectively.

For sonication, powdered triblock polymer was added to the bottom of a 50 ml conical tube. Water or 10mM HEPES + 150mM KCl, pH 7 was added to give a polymer concentration between 0.2 and 1 wt%. A 20 kHz 130 watt Ultrasonic Processor probe sonicator was used. Intensity ranged from 10% to 100% for 30 seconds to 15 minutes, depending on the experiment. Membrane labeling with DiI was conducted according to procedures for Vybrant Cell Labeling Solutions from Molecular Probes. Amicon spin filters were used to remove unbound dye.

For film hydration, triblock polymer was solubilized in EtOH at 1-4 mg/ml. PBD-PEO was solubilized in 9:1(v/v) chloroform-MeOH. 10-40ul drops were deposited on glass cover slips and dried overnight under vacuum. Hydration was performed with either water or 10mM HEPES + 150mM KCl, pH 7.

For protein reconstitution, purified protein (AqpZ or KvAP) was added to 0.3 wt% vesicles prepared through sonication to give a final concentration of 80-200 ug/ml. Samples were dialyzed overnight against three changes of buffer to remove detergent and drive incorporation. Alternatively, vesicles were incubated with detergent to partially solubilize vesicles prior to protein addition.

NTA-FITC labeling was done with a 10uM concentration of NTA-FITC in TBS buffer with an equivalent concentration of $NiSO_4$. Samples were then dialyzed against three buffer changes. Unincorporated AqpZ was removed using a 300 kD Amicon spin filter.

Procedures for labeling with Qdot-antiHis were modified from protocols supplied by Molecular Probes. Qdot conjugates fluorescing at 565nm were added at ~100nM solution after incubation with BSA for 20 minutes to minimize nonspecific binding. When necessary, excess antibody was removed with spin filters. The blot was performed as a standard Western blot.

Labeling with Monomaleimido Nanogold from Nanoprobes was performed prior to protein incorporation at a 3-6 nmol/ml concentrations of protein, with ~10-20 nmol/ml nanogold-maleimide reagent. Excess nanogold was removed using spin filters and protein was incorporated into polymer vesicles as explained above. Nanoprobes LM enhance solutions were added to samples before transfer to a flow cell, so growth of metal particles could be observed with DIC microscopy. Prior to enhancement, vesicles were incubated with 1% BSA to block nonspecific binding.

Samples were visualized using a disposable flow cell prepared with a slide and cover slip. All presented images were taken with an upright Nikon E800 microscope and a Hamamatsu 3CCD color camera with capture software written using National Instruments Labview.

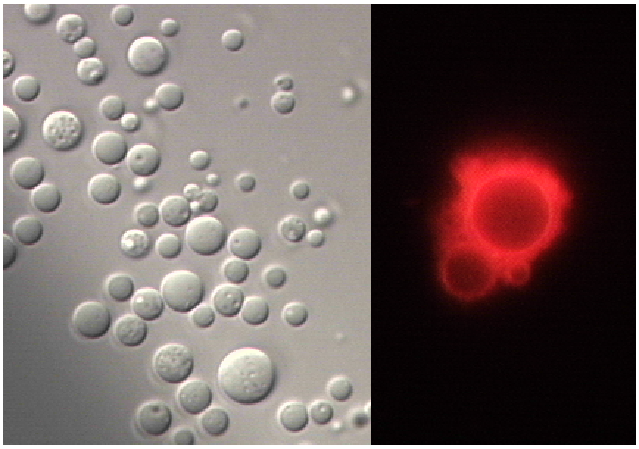

Figure 1. PMOXZ-PDMS-PMOXZ polymer vesicles formed through sonication. Left image, DIC. Right, fluorescent image of DiI labeled vesicles.

3 RESULTS AND DISCUSSION

3.1 Vesicle Formation through Sonication

These experiments used the long version of the PMOXZ-PDMS-PMOXZ triblock polymer. Micrometer-scale vesicles formed on some of the first attempts (figure 1). The addition of the carbocyanine dye, DiI highlighted the membrane in vesicles formed through sonication (figure 1). Binding was nonhomogeneous, with some vesicles heavily labeled and others undetectable with fluorescent microscopy.

We varied conditions to maximize vesicle size. Parameters explored included buffer composition, sonication time and intensity, and polymer concentration. Compared to vesicles prepared in water, vesicles prepared in HEPES-buffered KCl averaged smaller in size, and polymer aggregates were more commonly found in these solutions. Size appeared inversely proportional to sonication time—longer sonication produced more regular vesicles, reduced aggregates, and the population of vesicles trapped within other vesicles. A 30 sec, high intensity sonication produced very large vesicle (>50um) networks, as well as giant vesicles (figure 2). These preparations also contain a large number of vesicles in the ~10um size range. A long period of high intensity sonication produces a uniform population of submicron vesicles (data not shown).

Vesicles were readily prepared with relatively high concentrations of polymer ranging from 0.2-1 wt%. Larger numbers of vesicles were formed in more concentrated solutions. Evaporation proved effective for concentrating formed vesicles prior to visualization. As expected, large vesicles, such as those shown in figure 2, settled during storage.

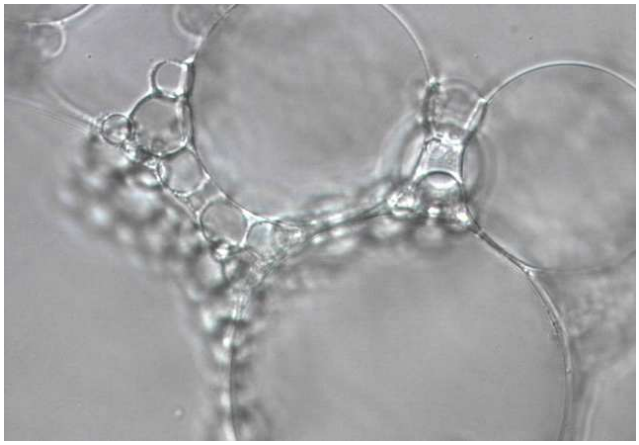

Figure 2. Network of giant vesicles formed by brief, high-intensity sonication.

Long sonications heated samples 10-20 ^{o}C. There did not to be appear any significant changes in vesicle formation when the sample was cooled during sonication. Factors such as agitation during the cooling process likewise did not seem to play a significant role in resulting vesicles. The formation process appears to be rapid and complete by the end of the sonication.

On one occasion fusion was observed between two giant vesicles prepared in Tris-buffered saline (TBS). Although vesicles were often seen aggregated or with non-vesicle polymer attached and sticking together (figure 1,2), this was the only instance of vesicle fusion observed. This seems to be somewhat unusual behavior, as an overall increase in vesicle size as time passed was not observed.

While a powerful technique, the major drawback of sonication is that, as visible in figure 1, the vesicles produced are nearly exclusively multilamellar. This makes them unsuitable for experiments observing movement across a membrane, such as ion transport by channel proteins using patch-clamp. These vesicles were, however, used for a number of protein incorporation studies.

3.2 Thin Film Hydration

Drying polymer solutions produced unique surface topologies depending on the type and concentration of polymer used. With the long triblock, multilamellar vesicles similar to those formed by sonication predominated, with a few unilamellar vesicles present. Films formed from the short triblock appeared similar to the long triblock, with rapidly formed vesicles observed but relatively scarce within the sample (figure 3). Both triblock polymers tended to detach from the slide surface without forming vesicles—the short polymer peeled off in curled sheets. On the other hand, the PBD-PEO diblock gradually formed large numbers of giant unilamellar vesicles (figure 3). As with the triblock polymers, different concentrations, drying conditions, and hydration solutions did little to change the production of vesicles.

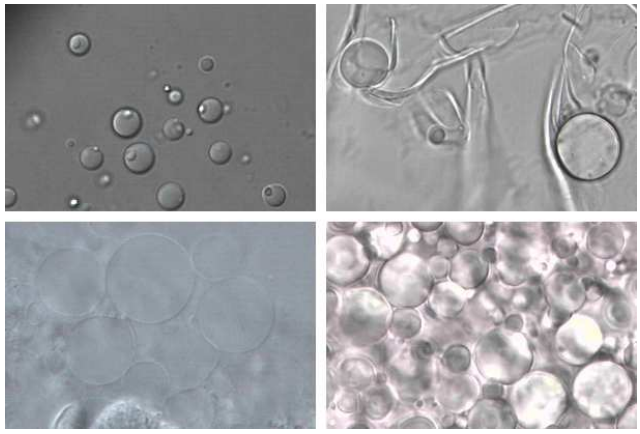

Figure 3. Vesicles formed by thin film hydration. Clockwise from top-left: long triblock, short triblock, phosphatidylcholine, PBD-PEO diblock.

The rehydration method seemed more effective with lipid and the PBD-PEO diblock polymer. One possible explanation is that triblock polymer did not form as regular layers as lipid or diblock does during the drying step. This would coincide with the observations that during rehydration variation was visible across the sample surfaces, with sheets of polymer, multilamellar vesicles, and aggregates predominating. It is possible that another solvent or surface other than glass would yield different results with the triblock polymer.

3.3 Fluorescent Labeling

When using fluorescence to study protein incorporation, it is difficult to generate enough localized emission for detection. We worked with both KvAP and AQPZ during the incorporation tests. The NTA-FITC tag did not demonstrate a strong affinity for vesicles or protein, but excess did prove difficult to remove, requiring extensive dialysis. This may have resulted from the separation of the relatively weak Ni-NTA bond.

Quantum dots coupled to an anti-histidine tag antibody have several advantages over Ni-NTA: they are brighter, less prone to bleaching, and should bind more strongly to proteins. We prepared polymer vesicles with and without incorporated KvAP. Incubation with Qdot-antiHis produced an unexpected result. Under fluorescent illumination, we were surprised to find that samples without added protein or Qdots fluoresced, indicating that the polymer itself was fluorescent.

An unusual feature of the polymer fluorescence was that it appeared to be a broad spectrum effect, occurring at every observed excitation wavelength. A sample of powdered polymer was examined and exhibited the same behavior. The fluorescence was more noticeable when larger amounts of polymer were present. Most of the vesicles had thin membranes and thus did not show significant fluorescence, but fluorescence was frequently observed in the

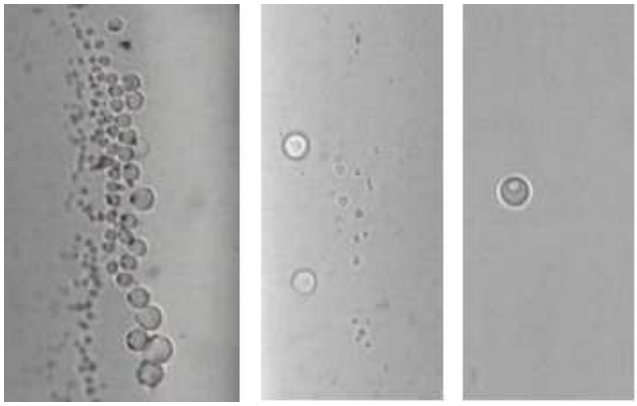

Figure 4. LM enhanced vesicles with KvAP prelabeled with Monomaleimido Nanogold. Left image: with incorporated KvAP. Center and right: control vesicles. Dark spots are enhanced gold particles bound to protein.

irregularities in vesicles—clumps attached to the surface or vesicles within vesicles.

We were unable to find the cause of the fluorescence, which was perhaps a remnant of synthesis if not a peculiarity of the triblock polymer itself. Regardless, the polymer fluorescence made it impossible to discriminate between vesicles with bound protein and false positives. The polymer fluorescence was brighter and larger than the Qdot tags, preventing the detection of the low intensity expected from reasonable protein incorporation. Fluorescence techniques were therefore incompatible with triblock polymer vesicles prepared through sonication.

3.4 Gold Labeling

Finally, proteins were labeled with gold nanoparticles prior to incorporation using Monomaleimido Nanogold, a 1.4 nm gold particle conjugated to a maleimide group. Light microscopy enhancers were used to increase the size of the gold particles immediately before observation. Early experiments made clear the necessity of removing unbound gold particles, as the enhancement reaction of excess nanogold quickly obscured the vesicles present in the sample.

In the presence of KvAP, the majority of the vesicles possessed one more dark nodules, indicating the presence of incorporated protein (figure 4). While significantly less common, a few control vesicles, prepared both with and without nanogold particles, exhibited the same structures. This appeared to be reduced by BSA, but suggests a degree of nonspecific binding of LM enhance reagent to the polymer itself.

4 CONCLUSION

Vesicles were succesfully formed from both PBD-PEO and PMOXZ-PDMS-PMOXZ polymers. Sonication, while not an ideal technique to prepare giant unilamellar vesicles, was quite effective at giant multilamellar vesicle formation. Multilamellar giant vesicles have found application as delivery systems able to stabilize hydrophobic compounds [2] and it is possible it would be useful in this context. Thin film hydration effectively produced giant unilamellar vesicles from the PBD-PEO diblock, making it a choice for future experiments applying patch-clamp to polymer membranes.

Polymer fluorescence creates interesting questions about the role of polymer autofluorescence for previous fluorometric studies completed with this polymer. With the presence of some nonspecific enhancement, it is difficult to hold the gold labeling results as irrefutable evidence for the presence of incorporated protein. However, given previous results and blot experiments performed (data not shown), it appears likely that this is the best evidence for protein incorporation in giant polymer vesicles obtainable through light microscopy.

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