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Stabilized Porous Phospholipid Nanoshells

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Abstract

Chemically stabilized, porous phospholipid nanoshells (PPNs) were prepared via copolymerization of reactive monomers with unilamellar bis-Sorbyl phosphatidylcholine vesicles. The resulting PPN vesicular assemblies possess a highly porous membrane structure that allows passage of small molecules, which can react with encapsulated proteins and reporters. The unique combination of membrane stability and porosity will prove useful for preparing nm-sized sensor, container and reactor platforms stable in harsh chemical and biological environments.

INTRODUCTION

Delivery of protein-based reporters and enzymes into primary cells and tissues markedly expands the toolbox available for intracellular chemical analysis and manipulation. Movement of proteins across the cell membrane is complicated by the large size of most proteins, generally requiring penetration of the membrane via microinjection or disruption of the membrane via liposomal or surfactant-based delivery systems. Though cell penetrating peptides provide efficient cellular delivery for a number of proteins and enzymes,¹ the utilization of this technology requires the generation of chimeric proteins, a time consuming and often arduous task. Preparation of viral and plasmid vectors allows endogenous expression of proteins and enzymes² but is typically used for immortalized tumor cell lines and not primary tissue and cell cultures. Moreover, preparation of viral and plasmid vectors requires substantial time and effort. Further complications arise from the short molecular lifetimes exhibited by translocated proteins due to the presence of proteases and other degradation pathways. Thus, there is a clear need to develop new technologies capable of simultaneously delivering proteins and enzymes into primary cells and increasing the overall stability of the payload once inside the cellular milieu.

Addressing these issues has fueled extensive efforts in designing biocompatible nanoscale materials useful for intracellular delivery.^{3, 4} Hollow nm-sized spheres (nanoshells) are particularly attractive due to their unique structural and materials properties, including the ability to encapsulate compounds in an aqueous core with minimized diffusional restrictions compared to solid polymeric particles. Markedly enhanced protection of encapsulated compounds from proteases and other biological insults can be achieved,⁵ while also protecting the cell from the nanoshell contents, thereby allowing delivery of potentially harmful components. Further, the incorporation of size-selective pores into the nanoshell allows small molecule analytes present on the nanoshell exterior to readily move across the nanoshell “membrane”, while preventing large molecular weight proteins and reporters encapsulated within the nanoshell from leaching, allowing construction of nanoshell reactors useful in the cellular environment.

The most common example of biocompatible nanoshells is a phospholipid vesicle, which contains a hydrophilic interior surrounded by a highly organized phospholipid bilayer,

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though nanoshells have been prepared from silica and a number of polymers as well.^{6, 7} Due to their simple preparation, biocompatibility and low toxicity,⁸ phospholipid vesicles have generated considerable interest as platforms for drug delivery,⁹ bioreactors and biosensors.^{10, 11} The nanoscale dimensions of phospholipid vesicles allow unparalleled reduction of confined volumes to the zL range, making them ideal for fabricating nanoreactor systems.¹⁰

Preparation of intracellular nanoreactors and nanocarriers requires that the membrane be sufficiently robust to retain the encapsulated species.^{12, 13} Phospholipid vesicles prepared using natural lipids lack the required chemical and physical stability for use as intracellular nanocarriers and nanoreactors.¹⁴ Further, phospholipid vesicle membranes are inherently impermeable to ions and hydrophilic small molecules, inhibiting analyte access to the nanoshell (vesicle) interior. A number of approaches have been introduced to enhance phospholipid vesicle stability including: polymerizable phospholipids,^{15–18} surface grafting of water soluble polymers¹⁹ and polymerization of hydrophobic monomer units incorporated into the bilayer.^{20–24} Preparation of phospholipid vesicles using polymerizable lipids presents a unique opportunity for preparing porous phospholipid nanoshells (PPNs) as the membrane fluidity, stability, and permeability can be readily altered by controlling the polymerization conditions.^{25–27} Here, we report the preparation and characterization of stabilized nm-sized porous shell structures for constructing nanoreactors and nanosensors that are stabilized for intracellular utilization.

EXPERIMENTAL

Materials

Bis-Sorbyl phosphatidylcholine (bis-SorbPC) was synthesized and purified using a modification to the procedure previously described.²⁸ Lipid purity was evaluated using thin-layer chromatography. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Ethylene glycol dimethacrylate (EGDMA) was obtained from Aldrich and passed through an inhibitor removal column (Aldrich) prior to use. Hemoglobin (Hb), 2,2-diethoxyacetophenone (DEAP), *o*-phthaldialdehyde (OPA), and 3-mercaptopropionic acid (3-MPA) were obtained from Sigma-Aldrich and used as received. Hexahistidine tagged enhanced green fluorescent protein (6xHis-EGFP) was prepared in house from bacterial colonies and purified using metal affinity chromatography. All experiments were performed in 10 mM borate buffer (pH 8.5) prepared in deionized (18.3 M Ω) water.

Vesicle Preparation

Unilamellar vesicles were prepared using the film hydration method.²⁹ Briefly, chloroform (DOPC, Rhodamine PE) or benzene (bis-SorbPC) was removed from the lipid stock solution (2 mg total lipid) using a direct stream of argon prior to vacuum desiccation for a minimum of four hours. The resultant dried lipid films were rehydrated with 2mL buffer for 30 minutes. Samples were subjected to ten freeze-thaw-vortex cycles in dry ice/2-propanol (-77°C) and warm H₂O (42°C), followed by extrusion 21 times through two stacked 100 nm Nuclepore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids).

For protein encapsulation, 200 μL of 150 mg/mL hemoglobin or 10 mg/mL EGFP was added to the dried lipid films and freeze-thaw and extrusion were performed as described. Non-entrapped protein was removed via size exclusion chromatography using Sepharose CL-4B (Sigma-Aldrich) and rehydration buffer as the eluent.

Vesicle Polymerization

Copolymerized vesicles were prepared as follows. A buffered solution of unilamellar extruded bis-SorbPC vesicles was mixed with the cross-linking agent EGDMA and the photoinitiator DEAP ($\lambda_{\text{max}} = 254 \text{ nm}$) at mole ratios of of 1:2 for [bis-SorbPC]/[EGDMA] and 10:1 for ([bis-SorbPC]+[EGDMA])/[DEAP]. The solution was stirred overnight at room temperature to maximize partitioning of the monomer and photoinitiator into the vesicle bilayer. The resulting vesicle solution was purged with argon to eliminate oxygen, and UV irradiated for 30 minutes using a UV pen lamp (UVP Pen-Ray Lamp, $\lambda = 254 \text{ nm}$). The solution was constantly stirred during photopolymerization to ensure homogeneous irradiation. Polymerized bis-SorbPC vesicles lacking EGDMA were prepared as described except EGDMA/DEAP was omitted.

Reaction of encapsulated hemoglobin with OPA/3-MPA

A 50 μL aliquot of LUVs (lipid concentration: 1 mg/mL) containing hemoglobin was added to 3 mL buffer containing 0.17 mM OPA/3-MPA and the reaction was allowed to proceed for 5 min with stirring at room temperature prior to collection of spectra. Solutions were excited at 335 nm and emission collected between 400 and 540 nm using a SPEX Fluorolog 2 spectrofluorimeter.

Cell Cultures and Particle Delivery

HeLa cells plated on microculture plates with glass coverslips forming the bottom were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum at 37 °C under 5% CO_2 . Cell culture media was replaced with 200 μL Krebs's Ringer Buffer (KRB) containing 0.05 mg/mL EGFP encapsulated PPNs and incubated for 1 h 37 °C under 5% CO_2 . Excess PPNs were removed via multiple KRB washes prior to collection of fluorescence images.

Fluorescence microscopy

Fluorescent images were collected on a Nikon Eclipse Quantum TE2000 with a 40 $\times$ /1.30 numerical aperture (NA) oil immersion objective and a Quantix 57 CCD camera (Roper Scientific, Tucson, AZ). For PPN stability studies, the concentration of bis-SorbPC and rhodamine PE was 0.2 mg/mL and $1.6 \times 10^{-3} \text{ mg/mL}$, respectively. Triton X-100 was added at [Triton X-100]/[lipid] mole ratio of 10. Excitation was by a 100 W mercury lamp with a D540/25 nm bandpass filter reflected by a 565DCLP dichroic mirror. Emission filter was a D620/60 nm bandpass. Exposure times were 100 ms.

Fluorescence microscopy of HeLa cells was performed as described with the exception of the filter cube which contained a D480/10 bandpass filter for excitation, a 505DCLP dichroic mirror and a 530/20 bandpass filter for emission.

Instrumentation

Dynamic light scattering (DLS) measurements were performed with a BI8000 autocorrelator (Brookhaven Instrument Corp., Holtsville, NY). The scattering angle was held constant at 90°. Transmission electron microscopy (TEM) was performed with a JEM-100CX II (JEOL) electron microscope operated at 80 kV.

RESULTS AND DISCUSSION

A schematic of stabilized porous phospholipid nanoshell (PPN) preparation is shown in Figure 1. Large unilamellar vesicles (LUVs) of approximately 100 nm diameter were prepared from bis-SorbPC phosphatidylcholine (bis-SorbPC) using established freeze-thaw

extrusion methods.²⁹ Following vesicle preparation, the hydrophobic crosslinker EGDMA, along with a UV-sensitive photoinitiator 2,2-diethoxyacetophenone (DEAP, $\lambda_{\text{max}} = 254$ nm), were added to the LUV suspension where they partition into the inner lamella of the hydrophobic LUV bilayer. The mixed bis-SorbPC and EGDMA monomers were then polymerized via UV-irradiation, forming a highly stabilized, cross-linked LUV. The mean diameter of the LUV was determined before and after polymerization using dynamic light scattering (DLS),³⁰ with no significant differences observed (105 ± 10 nm and ca. 110 ± 10 nm before and after polymerization, respectively, Figure 2A). The structure of stabilized PPNs was further investigated using transmission electron microscopy (TEM). Figure 2B shows the structural morphology of a typical PPN preparation. PPNs observed in the TEM image are approximately spherical in shape and appear to be unilamellar based on the lack of stained membranes in the PPN interior. Combined, these data indicate that no significant alteration in vesicle morphology occurs upon cross-linking polymerization.

The primary goal of preparing polymerized PPNs is to impart structural stability into biocompatible nanoshell architectures, thereby allowing intracellular delivery of proteins, enzymes and large molecular weight indicators and enhanced stability of the chemical payload. To further evaluate chemical and physical stability, PPN size was monitored in response to surfactant solubilization. LUVs prepared from native phospholipids dissolve to form mixed micelles with a mean diameter below 10 nm. Here, LUVs were prepared using either a) unpolymerized bis-SorbPC, b) UV-photopolymerized bis-SorbPC or c) UV polymerized bis-SorbPC with EGDMA and the mean diameter was measured by DLS in the presence and absence of Triton X-100. As seen in Figure 2C, unpolymerized bis-SorbPC LUVs are degraded beginning at a [Triton X-100]/[lipid] mole ratio of 1.5, and are completely destroyed by mole ratio 4.5. This value is typical for unpolymerized phospholipid vesicles, including LUVs prepared from DOPC (data not shown). UV-photopolymerized bis-SorbPC vesicles are marginally stabilized compared to unpolymerized bis-SorbPC requiring a 5.5 mole ratio of [Triton X-100]/[lipid] to completely degrade the vesicles. The slight improvement in PPN stability is due to the low degree of cross-linking that is obtained for UV-photopolymerized bis-SorbPC vesicles.³¹ Though a marginal improvement in stability was obtained using polymerizable phospholipids, the enhancement in stability is insufficient for preparing stabilized PPNs that are useful in intracellular environments. To improve the stability of PPNs, we incorporated EGDMA into the inner bilayer lamella of the bis-SorbPC vesicle at mole ratios of 2:1 for [EGDMA]/[bis-SorbPC] and 10:1 for ([bis-SorbPC]+[EGDMA])/[DEAP]. Preliminary studies showed that a ratio of [EGDMA]/[bis-SorbPC] < 2:1 is insufficient to stabilize the PPNs, and [EGDMA]/[bis-SorbPC] > 2:1 significantly increases the vesicle size (data not shown). The stability of the copolymer vesicles is markedly enhanced over bis-SorbPC vesicles as seen in Figure 2C, where addition of 5 mM Triton X-100 to the polymerized bis-SorbPC/EGDMA vesicles exerts no effect on the mean vesicle size beyond a [Triton X-100]/[lipid] mole ratio of 16.

To further explore the stabilization offered via copolymerization of PPNs, the surfactant stability of PPNs was monitored using high sensitivity fluorescence microscopy. Figure 3 shows the fluorescence images obtained for PPNs prepared from unpolymerized (Figure 3A and 3B) and polymerized (Figure 3C and 3D) bis-SorbPC/EGDMA, before (3A and 3C) and after (3B and 3D) addition of surfactant. In both cases, vesicles were prepared using 0.5 mole % Rhodamine-PE fluorescent lipid as a dopant. Prior to surfactant addition, a number of punctate fluorescence sources were observed for both unpolymerized and polymerized PPNs. Upon addition of Triton X-100 to final concentration 5 mM, the punctate fluorescence disappeared from the unpolymerized PPNs (Figure 3B) and the overall fluorescence background increased, indicative of PPN dissolution. In the polymerized PPN preparation, punctate fluorescence sources were observed at similar densities following surfactant addition (Figure 3D). These results clearly indicate that polymerization of the

PPN effectively stabilizes the nanostructure, suggesting that polymerized PPNs should remain intact upon crossing the cell membrane and in the intracellular environment. Although the exact chemical structure remains to be clarified, these data suggest the formation of a highly cross-linked network via copolymerization of bis-SorbPC with EGDMA.

To evaluate the porosity of the PPN membrane, as well as the potential for performing (bio)chemical reactions within PPNs, a rapid, fluorogenic reaction based on primary amine coupling with o-phthalaldehyde (OPA) in the presence of 3-mercaptopropionic acid (3-MPA) was utilized (Figure 4A). Water-soluble, high molecular weight primary amine containing compounds were encapsulated in vesicles. Specifically, hemoglobin was chosen as a model since it contains readily accessible primary amines on the surface and can be trapped in LUVs with high encapsulation efficiencies.³² Under the experimental conditions used here, the encapsulation efficiency was approximately 10 %, determined by size exclusion chromatography with UV absorbance detection. Furthermore, it was found that UV-irradiating hemoglobin does not affect the reaction with OPA/3-MPA (see Supporting Information).

Formation of a fluorescent species is expected only if all three of the reactants are present.³³ Therefore, introduction of OPA and the charged 3-MPA to the LUV exterior allows direct measurement of LUV permeability to small molecules. Hb-encapsulating LUVs were prepared from DOPC, unpolymerized bis-SorbPC, and polymerized bis-SorbPC/EGDMA and were added to a solution containing OPA/3-MPA and allowed to react for 5 min, at which time fluorescence spectra were acquired. Fluorescence intensities were normalized against the intensity obtained following surfactant dissolution of unpolymerized vesicles. The normalized spectra are shown in Figure 4B.

Prior to addition of Hb-encapsulating LUVs, buffer solution containing only OPA/3-MPA exhibited a fluorescence low background (1.1 %). The fluorescence increased to 14 % of the normalized value upon addition of Hb-encapsulating DOPC LUVs. The small magnitude of the increase is not unexpected since most common lipid bilayers exclude hydrophilic and/or charged chemical species from crossing the membrane. Combined, these results suggest that the DOPC membrane surrounding the encapsulated Hb impedes the ability of either OPA or 3-MPA, or both OPA and 3-MPA, to cross the membrane and react with Hb to generate a fluorescent product. Though both OPA and 3-MPA are soluble in aqueous solutions, 3-MPA is much more hydrophilic than OPA, is charged at the pH of the reaction and is therefore more likely to be excluded. The small increase observed may be due to diffusion of OPA across the DOPC bilayer with a free thiol in Hb contributing to the generation of fluorescent product.

In contrast to the inhibition observed by DOPC, unpolymerized, Hb-encapsulating bis-SorbPC LUVs show a 64% increase in the normalized fluorescence when added to OPA/3-MPA solution (Figure 4B). The substantial increase in fluorescence observed suggests that both OPA and 3-MPA readily diffuse through the bis-SorbPC membrane. LUVs prepared using DOPC are generally regarded as having a “defect” free or completely intact membrane. It has been shown that bis-SorbPC membranes are more permeable than DOPC, primarily by the inability to encapsulate small molecular weight components into the vesicle.²⁸ These results show clearly the ability of molecules residing in the vesicle exterior to cross the bis-SorbPC membrane and react with encapsulated large molecular weight compounds, e.g. Hb. This unusually high membrane permeability is likely due to the packing behavior of bis-SorbPC monomers, which contain an ester moiety in the phospholipid tail (Figure 1),³⁴ possibly coupled with a change in membrane fluidity imparted following polymerization.

Though unpolymerized bis-SorbPC PPNs provide the porosity necessary to construct membranes that are permeable to small molecules yet retain/exclude large molecular weight compounds, the lack of structural stability (Figure 2C) prevents their utilization in cellular environments. While copolymerization of bis-SorbPC/EGDMA provides a marked increase in the overall PPN stability, it is possible that the formation of the cross-linked polymer network may also affect membrane permeability. When Hb-encapsulating copolymerized bis-SorbPC/EGDMA LUVs were added to OPA/3-MPA solution, a 51% increase in normalized fluorescence intensity was observed (Figure 4B), indicating that the cross-linked membrane maintains a high permeability to OPA and 3-MPA. Thus it is apparent that stabilized, biocompatible PPNs can be prepared that maintain the high membrane permeability desired for a number of applications in chemical sensing, nanodelivery, etc.

To demonstrate the feasibility of utilizing PPNs to deliver proteins, enzymes and other large molecular weight components into living cells, we prepared PPNs with enhanced green fluorescent protein (EGFP) in the PPN interior. EGFP is a moderate size protein (27kDa) that is natively fluorescent, making it an ideal model protein. Figure 5 shows a series of transmission and fluorescence images of HeLa cells in the absence (Figure 5A and 5B) and presence (Figure 5C and 5D) of PPNs containing EGFP. Upon incubation with PPNs and subsequent removal via multiple washes, the fluorescence in the cells is markedly enhanced, suggesting internalization of the PPNs, likely via natural ingestions (endocytosis). HeLa cells readily internalize nanoparticles of similar size to those used in this work, including 120 nm phospholipid vesicles, 50 nm gold nanoparticles and 18 nm silica nanoparticles.^{35–37} Furthermore, EGFP fluorescence was stable within the cell in excess of two hours, suggesting that the PPN architecture protects the encapsulated components from intracellular proteases which may degrade the protein. Combined with PPN permeability and stability studies, these results strongly suggest the utility of polymerized PPNs for delivering enzymes and large molecular weight reporters to the intracellular environment and may represent a new paradigm for cellular analysis as well as diagnosis and treatment of disease.

CONCLUSION

We have shown that chemically and environmentally stabilized PPNs can be formed via copolymerization of bis-SorbPC and EGDMA. The nm-sized PPNs possess high permeability to small molecules while retaining/excluding large molecular weight compounds, e.g. proteins, enzymes, proteases, etc. Thus, by allowing small excluded molecules direct access to the contents of the PPN aqueous interior new paradigms in chemical delivery and chemical sensing can be envisioned. The unique combination of stability and permeability suggests the likelihood of using PPNs in a number of biologically and chemically important applications, e.g. biosensors, bioreactors, nanocatalysts, etc., in which target molecules can enter the aqueous core and react with the encapsulated components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic diagram of porous phospholipid nanoshells (PPNs). PPNs were fabricated by first preparing 100 nm or 200 nm bis-SorbPC vesicles using established phospholipid extrusion techniques. Following vesicle formation, ethylene glycol dimethacrylate (EGDMA) was partitioned into the inner lamellar region of the bis-SorbPC bilayer overnight. The resulting mixed monomer vesicles were polymerized via UV-irradiation.

Figure 2.

Characterization of PPV morphology and stability. A) Intensity-weighted size distribution of unpolymerized bis-SorbPC vesicles (○) and poly(bis-SorbPC)/EGDMA PPNs (●) in 10 mM borate buffer solution, pH 8.5. B) Transmission electron micrograph of poly(bis-SorbPC)/EGDMA vesicles. The sample was stained with ammonium molybdate to enhance the contrast between the lipid and aqueous domains. Scale bar represents 100 nm. C) Mean diameter of poly(bis-SorbPC)/EGDMA (▲) in the presence of Triton X-100 at 25° C. Both unpolymerized (■) and polymerized bis-SorbPC (▼) vesicles are shown for comparison.

Figure 3.

Epifluorescence images of rhodamine PE-doped unpolymerized and polymerized bis-SorbPC/EGDMA PPNs before and after addition of Triton X-100. A) Prior to addition of Triton X-100 single PPNs can be observed in the fluorescence image. B) Following addition of Triton X-100 to same vesicle suspension used in A), no individual PPNs are observed and the background fluorescence intensity increased. When polymerized PPNs are monitored before C) and after D) exposure to Triton X-100, individual PPNs were detected with no observable differences. Scale bar = 50 μm .

Figure 4.

Covalent labeling of PPN-encapsulated proteins. A) Fluorogenic reaction used to monitor vesicle permeability of stabilized PPNs. The amine reactive probe, α -phthalaldehyde (OPA) was used to label encapsulated proteins in the presence of 3-mercaptopropionic acid (3-MPA) in large unilamellar vesicles (LUVs) prepared from DOPC, unpolymerized bis-SorbPC and polymerized bis-SorbPC/EGDMA. B) Normalized fluorescence emission spectra of hemoglobin encapsulated into LUVs prepared with different lipid compositions upon exposure to OPA/3-MPA added to the LUV exterior. Spectra were obtained for: a.) OPA/3-MPA; b.) Hb-encapsulating DOPC with OPA/3-MPA; c.) Hb-encapsulating bis-SorbPC with OPA/3-MPA; d.) Hb-encapsulating Poly bis-SorbPC/EGDMA with OPA/3-MPA; and e.) the same as (b) and (c), but with addition of excess Triton X-100 to disrupt vesicles.

Figure 5. Cellular uptake of EGFP encapsulated in polymerized bis-SorbPC/EGDMA PPNs. A) Transmitted light image and B) fluorescence image of HeLa cells prior to addition of EGFP containing PPNs. C) Transmitted light image and D) fluorescence image of HeLa cells following one hour incubation with EGFP containing PPNs. Scale bar = 50 μm .