

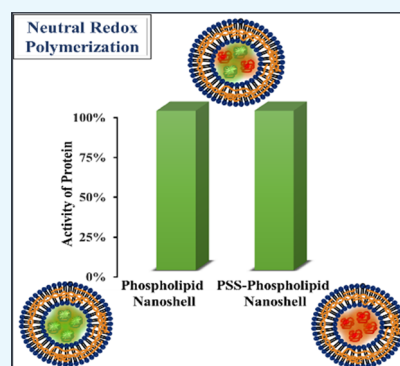
Enhanced Fluorescent Protein Activity in Polymer Scaffold-Stabilized Phospholipid Nanoshells Using Neutral Redox Initiator Polymerization Conditions

Surajit Ghosh,[†] Xuemin Wang,[†] Jinyan Wang,[†] Phuong-Diem Nguyen,[†] Colleen M. Janczak,[†] and Craig A. Aspinwall^{*,†,‡,§}

[†]Department of Chemistry and Biochemistry, [‡]BIO5 Institute, and [§]Department of Biomedical Engineering, The University of Arizona, Tucson, Arizona 85721, United States

Supporting Information

ABSTRACT: Phospholipid nanoshells, for example, liposomes, provide a versatile enabling platform for the development of nanometer-sized biosensors and molecular delivery systems. Utilization of phospholipid nanoshells is limited by the inherent instability in complex biological environments, where the phospholipid nanoshell may disassemble and degrade, thus releasing the contents and destroying sensor function. Polymer scaffold stabilization (PSS), wherein the phospholipid nanoshells are prepared by partitioning reactive monomers into the lipid bilayer lamella followed by radical polymerization, has emerged to increase phospholipid nanoshell stability. In this work, we investigated the effects of three different radical initiator conditions to fabricate stable PSS-phospholipid nanoshells yet retain the activity of encapsulated model fluorescent sensor proteins. To identify nondestructive initiation conditions, UV photoinitiation, neutral redox initiation, and thermal initiation were investigated as a function of PSS-phospholipid nanoshell stabilization and fluorescence emission intensity of enhanced green fluorescent protein (eGFP) and tandem dimer Tomato (td-Tomato). All three initiator approaches yielded comparably stable PSS-phospholipid nanoshells, although slight variations in PSS-phospholipid nanoshell size were observed, ranging from ca. 140 nm for unstabilized phospholipid nanoshells to 300–500 nm for PSS-phospholipid nanoshells. Fluorescence emission intensity of encapsulated eGFP was completely attenuated under thermal initiation (0% vs control), moderately attenuated under UV photoinitiation ($40 \pm 4\%$ vs control), and unaffected by neutral redox initiation ($97 \pm 3\%$ vs control). Fluorescence emission intensity of encapsulated td-Tomato was significantly attenuated under thermal initiation ($13 \pm 3\%$ vs control), moderately attenuated UV photoinitiation ($64 \pm 5\%$ vs control), and unaffected by neutral redox initiation ($98\% \pm 4\%$ vs control). Therefore, the neutral redox initiation method provides a significant advancement toward the preparation of protein-functionalized PSS-phospholipid nanoshells. These results should help to guide future applications and designs of biosensor platforms using PSS-phospholipid nanoshells and other polymer systems employing protein transducers.



INTRODUCTION

Biofunctionalized nanoarchitectures are increasingly utilized in biomedical applications,^{1–6} nanotechnology,^{7,8} biosensor development,^{9–12} and drug delivery.^{4,13–15} Self-assembled nanoarchitectures with a wide range of geometries, including liposomes, polymersomes, micelles, emulsions, and biofunctionalized metal nanoparticles, have been developed using phospholipids, polymers, and/or inorganic or hybrid materials.^{4,6} Among these promising nanoarchitectures, phospholipid nanoshells (liposomes) are attractive for intracellular sensing and drug delivery platforms because of the biocompatible nature of phospholipids and the ability to solubilize both hydrophilic and hydrophobic cargo, broadening their potential utility. Hydrophilic compounds are easily loaded into the aqueous lumen of the nanoshell, whereas the nanoshell bilayer serves as a natural carrier for hydrophobic materials. Phospholipid nanoshells can also be functionalized with

specific targeting ligands or with integral membrane proteins.^{15–19} The structural, morphological, and chemical similarities of phospholipid nanoshells with cell membranes enable encapsulation of various cargoes, including proteins, enzymes, and DNA molecules.^{16,20–24} Furthermore, the aqueous interior of the phospholipid nanoshell minimizes diffusional restrictions, which are sometimes encountered in solid and gel polymer nanoarchitectures, and enables the fabrication of bioinspired nanosensors and nanoreactors.^{25,26}

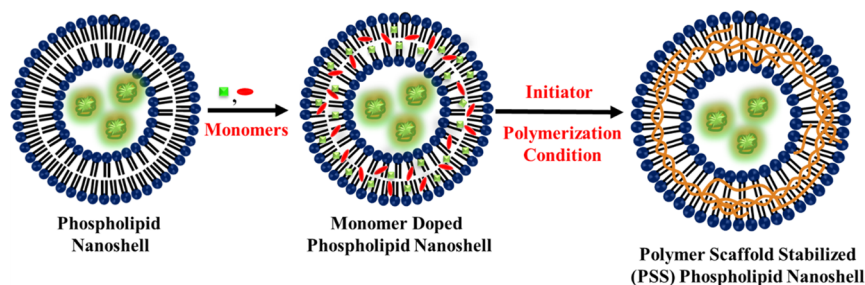
The application of phospholipid nanoshells in the fabrication of intracellular nanosensors and nanoreactors requires the ability to sufficiently stabilize the nanoshells while retaining activity of the encapsulated components. However, most

Received: July 15, 2018

Accepted: November 9, 2018

Published: November 26, 2018

Scheme 1. Schematic Representation of Polymer Scaffold Formation in Phospholipid Nanoshells



phospholipid nanoshells exhibit limited stability that hinders their use in cellular applications because of degradation and/or membrane fusion. Several approaches have been developed to enhance the stability of phospholipid nanoshells,^{22,27–33} including (but not limited to) the use of polymerizable lipids,^{28,30} polymerization of small hydrophobic monomers in the nanoshell bilayer,^{22,31} and surface grafting of water-soluble polymers.³² Polymerizable phospholipids are difficult to synthesize, and limited functionalities are available. Phospholipid nanoshells formed using polymerizable phospholipids may also exhibit significant leakage because of their packing behavior, which is problematic for long-term encapsulation of cargo.^{34–36} Alternatively, polymer scaffold-stabilized (PSS) phospholipid nanoshells can be fabricated using a range of readily available functionalized lipids. PSS-phospholipid nanoshells are formed via partitioning and subsequent polymerization of hydrophobic reactive monomers into the hydrophobic phospholipid bilayer lamella.^{22,31,37,38} This approach eliminates the requirement of polymerizable lipid synthesis, reduces the leakage of encapsulated molecules,¹⁰ and provides a promising route to increase the diversity and applicability of stabilized phospholipid nanoshells, particularly for biosensor platforms. On the basis of these properties, PSS-phospholipid nanoshells provide an attractive platform for development of nanosensors that can function in complex, harsh, and/or intracellular environments.

PSS-phospholipid nanoshell sensor platforms rely on the encapsulation of selective and sensitive reporter chemistries, including small molecules, enzymes, and fluorescent proteins. Previous investigations of stabilized nanoshells showed enhanced stability of encapsulated nucleic acids and small molecules.^{10,11} Fluorescent protein biosensors have evolved as analytical tools for measurement and/or visualization of specific analytes *in vitro* and *in vivo*.^{39–41} Two primary categories of fluorescent proteins with unique structural features have emerged for biological sensing and visualization, namely, DsRed, originally from marine corals, and green fluorescent protein (GFP), from jellyfish, each of which has been engineered into multiple variants that are useful across the entire visible spectrum and is sensitive to different environmental conditions.^{42–45} Encapsulation of fluorescent binding proteins into PSS-phospholipid nanoshells likely protects the proteins from nonspecific interferences and proteolytic degradation, thus enhancing sensor lifetime in complex chemical and biochemical environments. However, fabrication of the stabilizing polymer scaffold that promotes these benefits is most commonly performed using free-radical polymerization, primarily with UV photoinitiators or thermal initiators. The harsh chemical environments that result from high-intensity UV exposure and elevated temperature, as well as the high radical concentration, likely damage the function of

encapsulated reporter chemistries, particularly those that are based on fluorescent proteins. Therefore, it is necessary to optimize the polymerization conditions to retain the maximal function of encapsulated components.

This work describes a comparative study of fluorescent protein stabilities under different polymer initiation methods with the goal of identifying a series of polymerization conditions that lead to enhanced retention of activity. Two model proteins were utilized, enhanced GFP (eGFP) and tandem dimer Tomato (td-Tomato), GFP and Ds-Red homologs, which are broadly representative of fluorescent proteins used to prepare fluorescent sensors.

RESULTS AND DISCUSSION

The preparation of PSS-phospholipid nanoshell sensors and delivery architectures requires that the nanoshell is sufficiently stabilized to remain intact in harsh environments (UV exposure or elevated temperature) and thus retains the intended cargo. In general, radical polymerization is utilized for stabilization of PSS-phospholipid architectures, yet the conditions typically employed to generate the required radicals are likely to exert deleterious effects on encapsulated cargo function. In this work, we investigated a series of radical initiation conditions to identify a range of conditions that are compatible with retention of cargo function. Using different polymerization conditions to prepare PSS-phospholipid nanoshells, we sought to identify the optimal balance of phospholipid nanoshell stability and cargo function. PSS-phospholipid nanoshells and unstabilized controls were prepared using well-established freeze–thaw–extrusion methods with the common phospholipid DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine).⁴⁶ Following preparation, ethylene glycol dimethacrylate (EGDMA) and *n*-butyl methacrylate (BMA) monomers were partitioned into the bilayer of the nanoshells^{10,22} and subsequently polymerized (Scheme 1) using three different initiation methods (Irgacure 907; 2,2'-azobis(2-methylpropionamidine) dihydrochloride, AAPD; and $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{NaHSO}_3$).

Characterization of Phospholipid Nanoshell Morphology and Stabilization. To determine the effects of polymerization on phospholipid nanoshell morphology, the size of the unilamellar phospholipid nanoshells was monitored before and after the formation of polymer scaffolds by dynamic light scattering (DLS).⁴⁷ A significant change was observed in the size distribution profiles of phospholipid nanoshells after the formation of polymer scaffolds within the nanoshell bilayers (Figure S-1, Supporting Information). The formation of polymer scaffolds and the arrangement inside the bilayer lamella enhance the nanoshell rigidity.⁴⁸ Interestingly, a larger increase in the size of phospholipid nanoshells was observed

with neutral redox initiator- and thermal initiator-induced polymerization compared to photoinitiator-induced polymerization. The average diameter of unstabilized phospholipid nanoshells was $\sim 143 \pm 3$ nm [polydispersity index (PDI) = 0.11], whereas the average diameter of PSS-phospholipid nanoshells was $\sim 300 \pm 15$ nm (PDI = 0.25), $\sim 480 \pm 11$ nm (PDI = 0.20), and $\sim 340 \pm 12$ nm (PDI = 0.15) for photoinitiator-, neutral redox initiator-, and thermal initiator-induced polymerization, respectively. In the presence of the respective initiator, the monomer and cross-linker reacted to form polymer scaffolds inside the phospholipid nanoshell bilayer. The thermal initiator and neutral redox initiator couple is water-soluble, whereas the photoinitiator is not.

Previous reports of polymerized phospholipid nanoshells showed that hydrophilic initiators more effectively polymerize functionalities at the water–bilayer interface, whereas the hydrophobic initiators favor polymerization in the bilayer lamella.⁴⁹ Additionally, the size of polymerized phospholipid nanoshells prepared using polymeric lipids slightly increased after polymerization using hydrophobic photoinitiators.⁵ Several other investigations have noted similar results.^{3,27–29,47,49} In each of these cases, the monomer was embedded within the lipid molecule; thus, there were minimal changes in nanoshell size. Conversely, we observed increased nanoshell size when water-soluble initiators were used for PSS-phospholipid nanoshell stabilization compared to the minimal size changes when hydrophobic initiators were used. These data support an enhanced polymerization at the water–bilayer interface with hydrophilic initiators, which may lead to polymer protrusion from the PSS-phospholipid nanoshell and enhanced interactions, or possibly even cross-polymerization, between adjacent PSS-phospholipid nanoshells that result in the measurement of small aggregates that influence the size distribution. When UV photoinitiators are utilized, the polymer is contained to the nanoshell lamella, thus better indicating the size of individual nanoshells.

The morphology of the PSS-phospholipid nanoshell was further investigated by transmission electron microscopy (TEM). Negative staining TEM images (Figure S-2, Supporting Information) clearly indicate that PSS-phospholipid nanoshells are stable and almost spherical, irrespective of initiators. TEM images also indicate that the sizes of PSS-phospholipid nanoshells under neutral redox initiator- and thermal initiator-induced polymerization are comparably larger than that of photoinitiator-induced polymerization, correlating with DLS studies.

Unstabilized phospholipid nanoshells tend to fuse in a membrane-rich environment, which increases the possibility of degradation of the nanoshell architecture.⁵⁰ Hence, it is necessary to compare the stability of PSS-phospholipid nanoshells with that of unstabilized phospholipid nanoshells with a goal of optimizing the stability of the PSS-phospholipid nanoshell. To evaluate the stability of these nanoshells, the optical density of the phospholipid nanoshell solution was monitored in response to surfactant (TX-100) solubilization because surfactant molecules induce degradation of bilayer assemblies. The optical density, resulting from the scattering of nanoshell aggregates, of the phospholipid nanoshell solution was monitored at 630 nm, where phospholipid, monomers, or initiators do not absorb. Figure 1 shows the optical density of the phospholipid nanoshell solution at $[\text{TX-100}]/[\text{DOPC}] = 0$ (red) and $[\text{TX-100}]/[\text{DOPC}] = 10$ (green). The optical density of unstabilized phospholipid nanoshells approaches

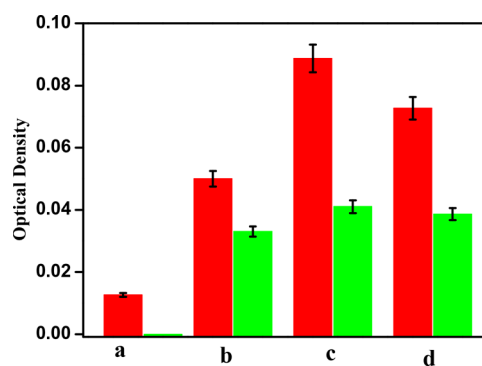


Figure 1. Variation in the optical density of unstabilized and PSS-phospholipid nanoshells at mole ratios of $[\text{TX-100}]/[\text{DOPC}] = 0$ (red) and $[\text{TX-100}]/[\text{DOPC}] = 10$ (green) in PBS buffer: (a) phospholipid nanoshells, (b) photoinitiator-induced PSS-phospholipid nanoshells, (c) redox initiator-induced PSS-phospholipid nanoshells, and (d) thermal initiator-induced PSS-phospholipid nanoshells. The concentration of DOPC and TX-100 in solution was 0.8 and 8.0 mM, respectively.

zero when $[\text{TX-100}]/[\text{DOPC}]$ is ~ 10 , supporting the hypothesis that surfactants can induce degradation of bilayer assemblies into small fragments that ultimately dissolved to form mixed micelles. Under all polymerization conditions, the optical density of the PSS-phospholipid nanoshell solution is higher than that of unstabilized nanoshells because of higher scattering efficiency of PSS-phospholipid nanoshells. The addition of excess TX-100 ($[\text{TX-100}]/[\text{DOPC}]$ ratio = 10) to PSS-phospholipid nanoshell solution results in a decrease, although to a nonzero value, because of the extraction of some phospholipid molecules from the polymer scaffolds by TX-100. These data support the hypothesis that formation of polymer scaffolds inside the phospholipid nanoshell bilayer leads to the stabilization, which hinders surfactant solubilization of the phospholipid nanoshells.

To further correlate the stability of PSS-phospholipid nanoshells in comparison with unstabilized phospholipid nanoshells, we also monitored the nanoshell size in the absence and presence of TX-100. Unstabilized DOPC nanoshells solubilize completely upon treatment of TX-100. The TX-100 dissolves unstabilized phospholipid nanoshells to form mixed micelles with an average diameter of ~ 10 nm (Figure S-1, Supporting Information) at $[\text{TX-100}]/[\text{DOPC}] = 10$. In contrast, upon treatment of PSS-phospholipid nanoshells with excess TX-100 at $[\text{TX-100}]/[\text{DOPC}] = 10$, an intense peak at higher diameters (~ 100 to 1000 nm) was observed. The variation in optical density and size of PSS-phospholipid nanoshells is likely due to the extraction of the lipid molecules from polymer scaffolds. The removal of a few phospholipids from polymer scaffolds in the presence of TX-100 may lead to aggregation of nanoshells containing hydrophobic polymer scaffolds, which is reflected in the DLS peak at a higher diameter range. Mixed micelles containing TX-100–DOPC were likely also present in solution, but the scattering intensity was markedly lower than that from the aggregated polymer scaffolds and thus was not detected by the DLS instrument. It should be noted that treatment of PSS-phospholipid nanoshells with excess TX-100 ($[\text{TX-100}]/[\text{DOPC}] = 10$) is an extremely harsh condition, which would not be encountered under typical utilization conditions. Therefore, the formation of polymer scaffolds should provide

sufficient stability to phospholipid nanoshells in the intracellular and other complex biological environments.

To confirm the formation of polymer scaffolds under each initiation condition in PSS-phospholipid nanoshells, we isolated the polymer scaffolds by washing the PSS-phospholipid nanoshells with methanol and water. SEM images of isolated polymer scaffolds are shown in Figure S-3 (Supporting Information). The images support aggregation of the polymer scaffolds after removal of phospholipids. Interestingly, photoinitiator-induced polymer scaffolds appeared to aggregate less than neutral redox initiator- and thermal initiator-induced polymer scaffolds.

Understanding the formation of polymer scaffolds and their influence on the phospholipid bilayer is crucial to understanding the effective stability of PSS-phospholipid nanoshells. Because PSS-phospholipid nanoshells formed under all three initiator conditions are stable in chemically harsh environments, these conditions may be used as a platform to design biosensors. However, the development of biosensors requires functional integration of sensing components, including proteins or enzymes, as well as nondestructive approaches to stabilize the sensor architecture that are compatible with sensor function.

Evaluation of Polymerization Conditions on Model Sensor Protein Activity. To utilize PSS-phospholipid nanoshells that encapsulate sensor proteins as platforms for biosensor development, the stability of the encapsulated proteins must be retained under polymerization conditions. The formation of polymer scaffolds in bilayers using an initiator requires the generation of excess free radicals as well as elevated temperature or UV irradiation. To evaluate the protein activity, eGFP and td-Tomato, fluorescent proteins that serve as model proteins for sensor design, were individually encapsulated in the aqueous core of phospholipid nanoshells using freeze–thaw–vortex–extrusion methods.⁵ In GFPs, the chromophore is surrounded by α helices and β sheets with an extensive hydrogen bonding network, which prevents the attack of water molecules that would quench fluorescence.⁵¹ The proper tertiary structure of eGFP is essential to exhibit fluorescence. The stability of fluorescent proteins is also sensitive to the surrounding environment and various factors, including salt concentrations, pH, and temperature.^{52–57} The radical polymerization conditions typically used produce free radicals in solution, as well as high doses of UV, high salt concentrations, or elevated temperatures, all of which may influence the structure and stability of eGFP and td-Tomato encapsulated in phospholipid nanoshells. Thus, variations in fluorescence intensity can be used as an indicator of protein stability, as it is directly related to fluorescence output.^{58,59}

To first evaluate the effects of the polymerization environment, phospholipid nanoshell-encapsulated eGFP and td-Tomato were exposed to UV irradiation/elevated temperatures, in the absence of radical initiators, and the fluorescence intensity of the protein was measured. The normalized emission spectra of eGFP and td-Tomato, and their relative changes after exposure to UV or elevated temperature (80 or 40 °C), are shown in Figures S-4 and S-5 (Supporting Information), respectively. The fluorescence spectra are normalized against the intensity of the respective protein, encapsulated in phospholipid nanoshells without polymerization treatment. The fluorescence spectra indicate that eGFP completely denatured at 80 °C, although it retained more than

90% fluorescence at 40 °C following UV irradiation. td-Tomato protein was relatively stable at elevated temperature, but the activity decreased significantly when exposed to UV irradiation compared to other conditions (80 or 40 °C). These results indicate that even in the absence of radical initiators, polymerization conditions may affect the activity of the encapsulated cargo.

Generation of PSS-phospholipid nanoshells further requires the partitioning of monomers and exposure to radical initiators. In addition to environmental conditions, the effects of monomer partitioning and increased radical concentrations due to the presence of radical initiators required for generation of PSS-phospholipid nanoshells were evaluated. The initial evaluation was performed under conditions where a monomer or initiator was excluded to prevent polymerization, allowing identification of the key contributors of degraded protein function. eGFP or td-Tomato was encapsulated in phospholipid nanoshells, and the resulting solution was divided into four aliquots. One aliquot was kept at 4 °C, and the second was exposed to UV light/elevated temperature (40 or 80 °C). To the remaining two aliquots, monomers or initiators were added, respectively, and then these solutions were exposed to UV light/elevated temperature (40 or 80 °C), individually. Finally, the fluorescence intensity of protein was measured in each aliquot. The fluorescence intensities were normalized against the intensity of protein encapsulated in phospholipid nanoshells kept at 4 °C. These data are summarized in Tables 1 and 2 and further described below.

Table 1. Activity of eGFP in Phospholipid Nanoshells under Different Initiator Conditions

polymerization conditions		relative fluorescence intensity (%) ^a		
initiation	conditions	nanoshells	monomer-doped nanoshells	initiator-exposed nanoshells
photo	UV, 0.5 h	95 ± 3	94 ± 4	64 ± 5
neutral redox	40 °C, 2 h	98 ± 2	98 ± 2	97 ± 3

^aRelative to eGFP encapsulated in phospholipid nanoshells with no treatment.

Table 2. Activity of td-Tomato in Phospholipid Nanoshells under Different Initiator Conditions

polymerization conditions		relative fluorescence intensity (%) ^a		
initiation	conditions	nanoshells	monomer-doped nanoshells	initiator-exposed nanoshells
photo	UV, 0.5 h	15 ± 4	13 ± 3	40 ± 4
neutral redox	40 °C, 2 h	99 ± 2	99 ± 4	98 ± 4
thermal	80 °C, 17 h	78 ± 5	77 ± 4	13 ± 3

^aRelative to td-Tomato encapsulated in phospholipid nanoshells with no treatment.

Before UV exposure, eGFP encapsulated in phospholipid nanoshells exhibits emission maxima at ~510 nm. The observed emission intensity of eGFP remains unaltered upon 0.5 h UV irradiation (Table 1 and Figure S-6, Supporting Information). In monomer-doped phospholipid nanoshells, the fluorescence intensity of eGFP was retained upon UV irradiation. A significant decrease in fluorescence intensity of eGFP was observed in initiator-doped phospholipid nanoshells

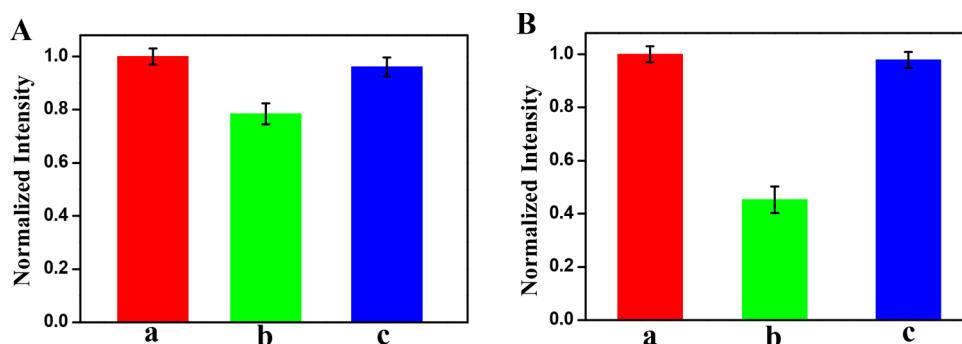


Figure 2. Relative fluorescence intensity of encapsulated (A) eGFP and (B) td-Tomato when encapsulated in phospholipid nanoshells before and after formation of polymer scaffolds using photoinitiator and neutral redox initiator. For each plot, the letter designations are as follows: (a) phospholipid nanoshells exposed to polymerizing conditions, but lacking initiator; (b) photoinitiator-induced PSS-phospholipid nanoshells; and (c) neutral redox initiator-induced PSS-phospholipid nanoshells. Fluorescence intensities are normalized to the corresponding encapsulation within unstabilized phospholipid nanoshells.

upon UV irradiation (Figure S-6A, Supporting Information), likely due to the generation of free radicals proximal to the eGFP. These results indicate that the activity of eGFP encapsulated in phospholipid nanoshells decreases under photoinitiated polymerization conditions. In contrast, the fluorescence intensity of eGFP encapsulated in the monomer-doped and neutral redox initiator-exposed phospholipid nanoshells remains unchanged compared to the controls (Table 1 and Figure S-6B, Supporting Information). Hence, these observations suggest that eGFP retains more than 90% fluorescence under neutral redox initiator-induced polymerization conditions. Thermal initiator-induced polymerization conditions were not evaluated for eGFP on the basis of the results of Figure S-4 (Supporting Information). Overall, eGFP fluorescence is retained at a significantly higher level (>95%) under neutral redox initiator-induced polymerization conditions than under photoinitiator-induced polymerization conditions (64%), suggesting that neutral redox initiator-induced polymerization provides the most effective initiation for eGFP.

Similarly, the stability of td-Tomato was evaluated under all three initiator conditions (Table 2 and Figure S-7, Supporting Information). The fluorescence intensity of td-Tomato encapsulated in phospholipid nanoshells decreased by 85% compared to controls upon UV irradiation (Figure S-7A, Supporting Information). The fluorescence intensity of td-Tomato encapsulated in monomer-doped phospholipid nanoshells also decreased by 87% compared to controls. However, in initiator-exposed phospholipid nanoshells, the emission intensity of td-Tomato decreased by only 60%. The observed decrease in fluorescence intensity of td-Tomato in initiator-doped phospholipid nanoshells likely results from the generation of free radicals from the initiator under UV irradiation that may alter the structure and decrease the activity of td-Tomato. In contrast, the fluorescence intensity of td-Tomato protein was unchanged in monomer- and initiator-doped phospholipid nanoshells incubated at 40 °C, the temperature required for neutral redox initiator-induced polymerization (Figure S-7B, Supporting Information). The emission intensity of td-Tomato encapsulated in phospholipid nanoshells exhibited a decrease upon exposure to thermal initiator-induced polymerization conditions, although the decrease associated with the temperature and monomer doping (22% and 23%, respectively) was significantly reduced compared to UV irradiation (Figure S-7C, Supporting

Information). Conversely, exposure to the initiator led to a larger decrease in emission intensity (87%) than in photoinitiator-induced polymerization (40%). Unlike eGFP, td-Tomato fluorescence is quite stable at elevated temperature (~80 °C). Thus, td-Tomato encapsulated in phospholipid nanoshells is moderately thermally stable but not stable in the presence of thermal initiators and the associated high radical concentrations.

The enhanced stability of fluorescent proteins encapsulated in phospholipid nanoshells observed under neutral redox conditions compared to thermal and UV irradiation likely results from a combination of the actual initiator and the condition required to generate radicals.^{35,60,61} The elevated temperature required for thermal initiation is uniform throughout the solution; thus, the encapsulated protein receives no protection from the phospholipid nanoshell. Additionally, the thermal initiator is a weak base, which may partition into the internal space of the phospholipid nanoshell, enabling direct reaction with the protein. Although UV photoinitiators are membrane-soluble and likely accumulate in the bilayer lamella, spatially restricting radical generation, the excess of hydroxyl radicals produced throughout the solution likely damages the protein. Thus, the milder, neutral redox polymerization, which yields low concentrations of membrane-impermeant anionic radicals, likely contributes to the enhanced stability observed.

To evaluate the stability of protein encapsulated in PSS-phospholipid nanoshells following polymerization with that of unstabilized phospholipid nanoshells, we measured the fluorescence intensity of eGFP and td-Tomato before and after formation of polymer scaffolds. As the thermal initiator method was deemed not suitable for preparation of PSS-phospholipid nanoshells that encapsulate functional proteins, we compared the stability of both proteins under photoinitiator- and neutral redox initiator-induced PSS-phospholipid nanoshell formation. The relative changes in normalized intensity of encapsulated eGFP and td-Tomato before and after formation of polymer scaffolds using photoinitiator and neutral redox initiator are shown in Figure 2. Both eGFP and td-Tomato exhibit >90% fluorescence intensity of control in neutral redox initiator-induced PSS-phospholipid nanoshells, although the activity decreases moderately (20% for eGFP and 50% for td-Tomato) under photoinitiator-induced polymerization.

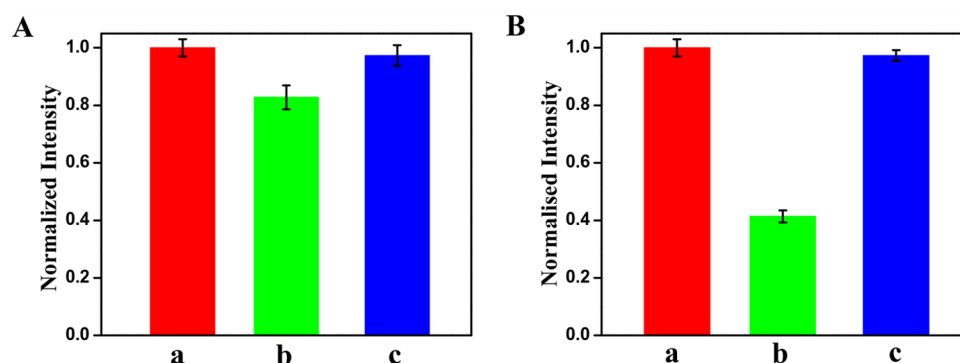


Figure 3. Relative change in fluorescence intensity of encapsulated (A) eGFP and (B) td-Tomato when co-encapsulated in phospholipid nanoshells before and after formation of polymer scaffolds using photoinitiator and neutral redox initiator. For each plot, the letter designations are as follows: (a) phospholipid nanoshells exposed to polymerizing conditions, but lacking initiator; (b) photoinitiator-induced PSS-phospholipid nanoshells; and (c) neutral redox initiator-induced PSS-phospholipid nanoshells. Fluorescence intensities are normalized to the corresponding encapsulation within unstabilized phospholipid nanoshells.

To further evaluate whether the eGFP and td-Tomato encapsulated in PSS-phospholipid nanoshells are suitable for microscopic studies, fluorescence images were obtained using an epifluorescence microscope. Figures S-8 and S-9 (Supporting Information) show images of eGFP and td-Tomato encapsulated in PSS-phospholipid nanoshells prepared using photoinitiator and neutral redox initiator and subsequently deposited onto glass slides. In each image, several punctate fluorescence sources were detected. The eGFP and td-Tomato encapsulating PSS-phospholipid nanoshells were observed as discrete spots that were highly fluorescent and observed through multiple image frames. Unstabilized phospholipid nanoshells have the tendency to fuse on glass surfaces⁶² and subsequently release and dilute the encapsulated species, making detection of punctate sources difficult. The presence and long-term observation of the corresponding PSS-phospholipid nanoshells further support the enhanced stability afforded by the polymer scaffold and enable more reliable observation of individual nanoshells.

Co-loading of eGFP and td-Tomato in Phospholipid Nanoshells. To demonstrate the possibility of designing multicolor biosensors using PSS-phospholipid nanoshells and evaluate the simultaneous effects on protein activity, eGFP and td-Tomato were co-encapsulated in PSS-phospholipid nanoshells. Co-encapsulation of both proteins was confirmed by size exclusion chromatography (SEC) (Figure S-10, Supporting Information), which shows that both proteins elute at the same volume with an effective separation from free proteins. To further investigate the effects of co-encapsulation on fluorescence intensity, the fluorescence intensity of eGFP and td-Tomato co-encapsulated in phospholipid nanoshells was measured under photoinitiator and neutral redox initiator polymerization conditions (Figure S-11, Supporting Information). Under photoinitiator and neutral redox initiator conditions, the relative change in intensity is similar to that of protein encapsulated in phospholipid nanoshells separately (Tables 1 and 2 and Figures S-6 and S-7, Supporting Information).

We then measured the fluorescence intensity of both proteins co-encapsulated in PSS-phospholipid nanoshells, before and after formation of polymer scaffolds (Figure 3). The observed reductions in fluorescence intensity for the co-encapsulated proteins were equivalent to the values for individual encapsulation, as shown in Figure 2. Therefore,

eGFP and td-Tomato exhibit similar trends in retention of activity in PSS-phospholipid nanoshells after individual encapsulation and co-encapsulation.

Finally, Figure 4 shows a series of fluorescence images collected using emission filters for eGFP and td-Tomato in PSS-phospholipid nanoshells. In these images, only PSS-nanoshells that contain at least one fluorescent protein can be

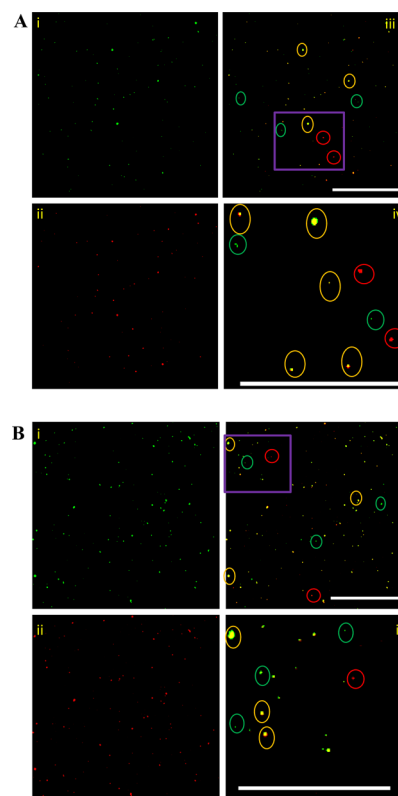


Figure 4. Epifluorescence images of eGFP and td-Tomato co-encapsulated in PSS-phospholipid nanoshells prepared using (A) photoinitiator and (B) neutral redox initiator. Images are shown in false color and were collected using a (i) green filter or (ii) red filter and further displayed as (iii) the merged image. (iv) Enlarged view of the area shown in the purple boxes in image iii for both (A) and (B). The green, red, and yellow circles indicate eGFP, td-Tomato, and co-encapsulated protein in PSS-phospholipid nanoshells, respectively. Scale bar = 50 μm .

visualized. Punctate fluorescence sources were observed corresponding to eGFP and td-Tomato encapsulated individually in PSS-phospholipid nanoshells, as well as co-encapsulation of the proteins. An expanded view of part iii is shown in part iv for Figure 4A,B, which more clearly shows the presence of individual protein loaded and co-loaded PSS-phospholipid nanoshells. Empirically, PSS-phospholipid nanoshells prepared using UV photoinitiator showed lower percentages of nanoshells containing only DsRed than those prepared with neutral redox initiators, agreeing well with bulk measurements in Figure 3. Interestingly, a higher fraction of nanoshells that contained both proteins was observed than predicted on the basis of bulk measurements (ca. 50% for each initiator, compared to ca. 25% expected for random distributions), although the nature of this observation is unknown. Combined, these observations suggest that co-encapsulation does not negatively affect fluorescence intensity and that individual protein-encapsulating PSS nanoshells can be observed. Furthermore, these measurements demonstrate the benefits of using proper initiation methods for preparing stabilized PSS-phospholipid nanoshells for co-encapsulation of sensor components.

CONCLUSIONS

PSS-phospholipid nanoshells were prepared by partition and subsequent polymerization of hydrophobic methacrylate monomers in the presence of photoinitiator, neutral redox initiator, and thermal initiator, individually. Polymerization induced by each type of initiator was sufficient to stabilize the PSS-phospholipid nanoshell to harsh chemical environments, and generally, all three initiation approaches provided equivalent stabilization, with minor differences in the size of the resulting PSS-phospholipid nanoshells. Investigation of the fluorescence activity of encapsulated model fluorescent proteins revealed substantial differences between each initiation pathway. The neutral redox initiation approach yielded the maximum retention of fluorescence activity for both eGFP and td-Tomato, whereas photoinitiation and thermal initiation resulted in approximately equivalent stabilization. This work clearly demonstrates the importance of optimizing polymerization conditions for polymeric, biofunctionalized nanomaterials, is broadly applicable beyond the design of PSS-phospholipid nanoshells, and demonstrates the feasibility of a new neutral radical polymerization initiation approach.

EXPERIMENTAL SECTION

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 2-Methyl-4'-(methylthio)-2-morpholinopropiophenone (Irgacure 907), 2,2'-azo-bis(2-methylpropionamidine) dihydrochloride (AAPD), $(\text{NH}_4)_2\text{S}_2\text{O}_8$, NaHSO_3 , ethylene glycol dimethacrylate (EGDMA), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Uranyl acetate and *n*-butyl methacrylate (BMA) were purchased from Electron Microscopy Sciences and Alfa Aesar (Ward Hill, MA), respectively. Aluminum oxide (neutral, 50–200 μm) was purchased from Acros Organics (Morris, NJ). The structures of DOPC, BMA, EGDMA, Irgacure 907, and AAPD are shown in Scheme S-1 (Supporting Information).

Hexahistidine-tagged eGFP and td-Tomato were expressed and purified in-house. Both proteins were expressed in

BL21(DE3) competent *Escherichia coli* (Thermo Fisher Scientific, Waltham, MA) and purified with TALON metal affinity resins (Clontech Laboratories, Inc., Mountain View, CA).

All experiments were performed in 10 mM phosphate-buffered saline (PBS) (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and prepared with deionized water (18 M Ω cm).

Preparation of Phospholipid Nanoshells. Unilamellar phospholipid nanoshells were prepared by film hydration followed by freeze-thaw and extrusion.⁴⁶ Briefly, 10 mg of DOPC in CHCl_3 was dried with Ar and further vacuum-dried for ≥ 4 h to completely remove trace CHCl_3 . Dried lipid films were rehydrated using 1 mL of PBS buffer (pH 7.4). The lipid solution was flushed with Ar, warmed to 42 $^\circ\text{C}$, and vortexed gently to resuspend the lipid. The lipid solution then went through 10 cycles of freeze–thaw–vortex in isopropanol–dry ice (-77 $^\circ\text{C}$) and warm water (42 $^\circ\text{C}$). Finally, the resulting solution was extruded 21 times through two stacked Nuclepore polycarbonate membrane filters (200 nm) using a stainless steel extruder (Avanti Polar Lipids, Birmingham, AL). The extruded phospholipid nanoshell solution was then diluted with PBS (pH 7.4) to a total volume of 5 mL (final DOPC concentration = 2 mg/mL).

Formation of Polymer Scaffolds in Phospholipid Nanoshells. PSS-phospholipid nanoshells were prepared using BMA and EGDMA, with three different initiation methods: (a) Irgacure 907 photoinitiator, (b) AAPD thermal initiator, and (c) $(\text{NH}_4)_2\text{S}_2\text{O}_8$ / NaHSO_3 neutral redox initiator pair. The monomer to initiator mole ratio was maintained at 5:1 in all polymerizations. Briefly, ~ 14 μmol of BMA and ~ 11 μmol of EGDMA were added to freshly prepared phospholipid (5 mL; 2 mg/mL) nanoshells. The solution was stirred overnight in the dark at 24 ± 2 $^\circ\text{C}$ to maximize the partitioning of hydrophobic monomers into the bilayer lamella. The monomer-doped phospholipid nanoshell solution was then polymerized using the different initiators as described below.

Photoinitiation. Irgacure 907 was dissolved in methanol at 10 mg/mL immediately before use. The required amount (monomer to initiator mole ratio = 5:1) of initiator stock solution was transferred into a glass vial, dried with Ar, and lyophilized for ~ 1.5 to 2 h. Monomer-doped nanoshell was added and stirred in the dark to complete partitioning. The monomer- and initiator-doped nanoshell solution was purged with Ar for 10 min and then polymerized with a 100 W Hg arc lamp for 0.5 h. The solution was constantly stirred during polymerization to ensure homogeneous exposure.

Thermal Initiation. AAPD was recrystallized in water and dried under vacuum. The recrystallized AAPD was dissolved in PBS buffer at 10 mg/mL immediately before use. AAPD (monomer to initiator mole ratio = 5:1) was added to the monomer-doped phospholipid nanoshell solution. The suspension was degassed with Ar for 10 min and heated at 80 $^\circ\text{C}$ in the dark for 17 h with continuous stirring to initiate polymerization.

Neutral Redox Initiation. $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and NaHSO_3 are used as neutral redox initiator pair.⁶³ $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and NaHSO_3 were dissolved separately in PBS buffer at 10 mg/mL, each immediately before use. $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and NaHSO_3 solution (monomer to initiator mole ratio = 5:1) were added to the monomer-doped phospholipid nanoshell solution. The suspension was degassed with Ar for 10 min and heated at 40

°C in the dark for 2 h with continuous stirring to initiate polymerization.

Isolation of Polymer Scaffolds. To isolate the polymer scaffold from phospholipid nanoshells, the phospholipid was removed by washing the PSS-phospholipid nanoshells with methanol. Initially, methanol was added to the aqueous suspension of PSS-phospholipid nanoshells and centrifuged to precipitate the polymer scaffolds. The precipitate was then resuspended in methanol and washed repeatedly with methanol to remove DOPC completely. Finally, the isolated polymer scaffolds were washed with H₂O and freeze-dried. Because methanol is used to break the phospholipid nanoshells⁶⁴ and phospholipids are completely soluble in methanol, the isolated precipitates are most likely composed of polymer scaffolds. The isolated polymer scaffold was then dispersed in methanol for analysis by SEM.

Encapsulation of Fluorescent Proteins in Phospholipid Nanoshells. The encapsulation of fluorescent proteins, eGFP and td-Tomato, into unstabilized and stabilized phospholipid nanoshells was performed by 10 cycles of freeze–thaw–vortex in isopropanol–dry ice (−77 °C) and warm water (42 °C), followed by extrusion and SEC.⁵ During protein encapsulation, eGFP and/or td-Tomato dissolved in PBS was added to dried DOPC films. Initially, the protein-containing lipid solution was warmed to 42 °C for 2 min and vortexed to resuspend the lipid thoroughly. Finally, the solution was exposed to freeze–thaw–vortex cycles as described. Unencapsulated protein was removed by SEC using a Sepharose CL-4B column (Sigma-Aldrich) and PBS buffer as eluent. The encapsulation efficiency was 20% ± 8% and 17% ± 6% for eGFP and td-Tomato, respectively. Similarly, during co-loading, the encapsulation efficiency was 17 ± 7% and 12 ± 5% for eGFP and td-Tomato, respectively. PSS-phospholipid nanoshells were prepared by partitioning monomers (BMA and EGDMA) into the bilayer of phospholipid nanoshells and subsequently polymerized using the experimental initiation methods.

Characterization of PSS-Phospholipid Nanoshells. DLS was used to measure phospholipid nanoshell sizes. DLS measurement of phospholipid nanoshells and PSS-phospholipid nanoshell size was performed using a Malvern Zetasizer Nano ZS (Worcestershire, United Kingdom) with a back-scattering angle of 173°. The measurements were made for each sample at 24 ± 2 °C.

The optical density of nanoshell solutions was measured by a Thermo Fisher Scientific NanoDrop 2000c spectrophotometer at 630 nm using a cuvette with a 1 cm path length.

The fluorescence of eGFP and td-Tomato was analyzed using a PTI QuantaMaster fluorescence spectrophotometer. Fluorescence intensities were monitored by excitation of the samples at 488 nm (for eGFP) and 554 nm (for td-Tomato).

TEM images were collected using a Tecnai G2 Spirit transmission electron microscope. Uranyl acetate (0.3 wt %) was used as the staining agent of PSS-phospholipid nanoshells.

SEM measurement of polymer scaffolds was performed using an FEI Inspec-S SEM equipped with a standard imaging backscattered electron detector, an energy-dispersive X-ray spectroscopy (EDS) system, and a JC Nabity Nanometer Pattern Generation System.

Protein-encapsulated PSS-phospholipid nanoshells were imaged using a Hamamatsu ImagEM X2 digital camera attached to a Nikon Eclipse TE300 Quantum inverted epifluorescence microscope. Emission was collected through

an oil-immersion objective (40×, numerical aperture = 1.30), with a filter cube (green filter, 540 nm/25 nm; red filter, 620 nm/60 nm). HCS Image Live software was used to collect the images, and ImageJ software was used to analyze the images.⁶⁵

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01661.

Structures of monomers and initiators, additional characterization data including DLS profiles, TEM images, SEM images, fluorescence spectra and normalized intensities of eGFP and td-Tomato, and epifluorescence images (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: aspinwal@email.arizona.edu (C.A.A.). Phone: 520-621-6338. Fax: 520-621-8407.

ORCID

Craig A. Aspinwall: 0000-0002-4359-5812

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research work was supported by the National Institutes of Health under Award Numbers R01EB007047, R01GM116946, and R01GM095763. All SEM images were collected in the W.M. Keck Center for Nano-Scale Imaging in the Department of Chemistry and Biochemistry at the University of Arizona with funding from the W.M. Keck Foundation Grant. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

■ REFERENCES

- (1) Lee, Y. E.; Smith, R.; Kopelman, R. Nanoparticle PEBBLE Sensors in Live Cells and In Vivo. *Annu. Rev. Anal. Chem.* **2009**, *2*, 57–76.
- (2) Gao, D.; Xu, H.; Philbert, M. A.; Kopelman, R. Bioeliminable Nanohydrogels for Drug Delivery. *Nano Lett.* **2008**, *8*, 3320–3324.
- (3) Cheng, Z.; Zaki, A. A.; Jones, I. W.; Hall, H. K.; Aspinwall, C. A.; Tsourkas, A. Stabilized Porous Liposomes with Encapsulated Gd-Labeled Dextran as a Highly Efficient MRI Contrast Agent. *Chem. Comm.* **2014**, *50*, 2502–2504.
- (4) Palivan, C. G.; Goers, R.; Najer, A.; Zhang, X.; Meier, W. Bioinspired Polymer Vesicles and Membranes for Biological and Medical Applications. *Chem. Soc. Rev.* **2016**, *45*, 377–411.
- (5) Cheng, Z.; D'Ambruso, G. D.; Aspinwall, C. A. Stabilized Porous Phospholipid Nanoshells. *Langmuir* **2006**, *22*, 9507–9511.
- (6) Turner, B. J. L.; Pan, D.; Plummer, R.; Chen, Z.; Whittaker, A. K.; Wooley, K. L. Synthesis of Gadolinium-Labeled Shell-Crosslinked Nanoparticles for Magnetic Resonance Imaging Applications. *Adv. Funct. Mater.* **2005**, *15*, 1248–1254.
- (7) Janczak, C. M.; Aspinwall, C. A. Composite Nanoparticles: The Best of Two Worlds. *Anal. Bioanal. Chem.* **2012**, *402*, 83–89.
- (8) Zamborini, F. P.; Bao, L.; Dasari, R. Nanoparticles in Measurement Science. *Anal. Chem.* **2012**, *84*, 541–576.
- (9) Sahari, A.; Ruckh, T. T.; Hutchings, R.; Clark, H. A. Development of an Optical Nanosensor Incorporating a pH-Sensitive Quencher Dye for Potassium Imaging. *Anal. Chem.* **2015**, *87*, 10684–10687.

- (10) Cheng, Z.; Aspinwall, C. A. Nanometre-Sized Molecular Oxygen Sensors Prepared from Polymer Stabilized Phospholipid Vesicles. *Analyst* **2006**, *131*, 236–243.
- (11) Li, Z.; Muhandiramlage, T. P.; Keogh, J. P.; Hall, H. K., Jr.; Aspinwall, C. A. Aptamer-Functionalized Porous Phospholipid Nanoshells for Direct Measurement of Hg^{2+} in Urine. *Anal. Bioanal. Chem.* **2015**, *407*, 953–960.
- (12) Xu, H.; Aylott, J. W.; Kopelman, R.; Arbor, A. Fluorescent Nano-PEBBLE Sensors Designed for Intracellular Glucose Imaging. *Analyst* **2002**, *127*, 1471–1477.
- (13) Chen, G.; Roy, I.; Yang, C.; Prasad, P. N. Nanochemistry and Nanomedicine for Nanoparticle-Based Diagnostics and Therapy. *Chem. Rev.* **2016**, *116*, 2826–2885.
- (14) Cheng, Z.; Elias, D. R.; Kamat, N. P.; Johnston, E. D.; Poloukhine, A.; Popik, V.; Hammer, D. A.; Tsourkas, A. Improved Tumor Targeting of Polymer-Based Nanovesicles Using Polymer λ Lipid Blends. *Bioconjugate Chem.* **2011**, *22*, 2021–2029.
- (15) Pattni, B. S.; Chupin, V. V.; Torchilin, V. P. New Developments in Liposomal Drug Delivery. *Chem. Rev.* **2015**, *115*, 10938–10966.
- (16) Vamvakaki, V.; Fournier, D.; Chaniotakis, N. A. Fluorescence Detection of Enzymatic Activity within a Liposome Based Nano-Biosensor. *Biosens. Bioelectron.* **2005**, *21*, 384–388.
- (17) Jesorka, A.; Orwar, O. Liposomes: Technologies and Analytical Applications. *Annu. Rev. Anal. Chem.* **2008**, *1*, 801–832.
- (18) Torchilin, V. P.; Rammohan, R.; Weissig, V.; Levchenko, T. S. TAT Peptide on the Surface of Liposomes Affords Their Efficient Intracellular Delivery Even at Low Temperature and in the Presence of Metabolic Inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 8786–8791.
- (19) Schmitt, C.; Lippert, A. H.; Bonakdar, N.; Sandoghdar, V.; Voll, L. M. Compartmentalization and Transport in Synthetic Vesicles. *Front. Bioeng. Biotechnol.* **2016**, *4*, 19.
- (20) Stano, P.; Carrara, P.; Kuruma, Y.; de Souza, T. P.; Luisi, P. L. Compartmentalized Reactions as a Case of Soft-Matter Biotechnology: Synthesis of Proteins and Nucleic Acids inside Lipid Vesicles. *J. Mater. Chem.* **2011**, *21*, 18887–18902.
- (21) Hamada, T.; Fujimoto, R.; Shimobayashi, S. F.; Ichikawa, M.; Takagi, M. Molecular Behavior of DNA in a Cell-Sized Compartment Coated by Lipids. *Phys. Rev. E* **2015**, *91*, 062717.
- (22) Graff, A.; Winterhalter, M.; Meier, W. Nanoreactors from Polymer-Stabilized Liposomes. *Langmuir* **2001**, *17*, 919–923.
- (23) Zhang, Y.; Schmid, Y. R. F.; Luginbu, S.; Wang, Q.; Dittrich, P. S.; Walde, P. Spectrophotometric Quantification of Peroxidase with *p*-Phenylene-diamine for Analyzing Peroxidase-Encapsulating Lipid Vesicles. *Anal. Chem.* **2017**, *89*, 5484–5493.
- (24) Cisse, I.; Okumus, B.; Joo, C.; Ha, T. Fueling Protein-DNA Interactions inside Porous Nanocontainers. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 12646–12650.
- (25) Nishimura, K.; Matsuura, T.; Nishimura, K.; Sunami, T.; Suzuki, H.; Yomo, T. Cell-Free Protein Synthesis inside Giant Unilamellar Vesicles Analyzed by Flow Cytometry. *Langmuir* **2012**, *28*, 8426–8432.
- (26) Elani, Y.; Law, V.; Ces, O. Protein Synthesis in Artificial Cells: Using Compartmentalisation for Spatial Organisation in Vesicle Bioreactors. *Phys. Chem. Chem. Phys.* **2015**, *17*, 15534–15537.
- (27) Regen, S. L.; Czech, B.; Singh, A. Polymerized Vesicles. *J. Am. Chem. Soc.* **1980**, *102*, 6638–6640.
- (28) Liu, S.; Brien, D. F. O. Stable Polymeric Nanoballoons: Lyophilization and Rehydration of Cross-Linked Liposomes. *J. Am. Chem. Soc.* **2002**, *124*, 6037–6042.
- (29) Lee, J.; Danino, D.; Raghavan, S. R. Polymerizable Vesicles Based on a Single-Tailed Fatty Acid Surfactant: A Simple Route to Robust Nanocontainers. *Langmuir* **2009**, *25*, 1566–1571.
- (30) Gallagher, E. S.; Mansfield, E.; Aspinwall, C. A. Stabilized Phospholipid Membranes in Chromatography: Toward Membrane Protein-Functionalized Stationary Phases. *Anal. Bioanal. Chem.* **2014**, *406*, 2223–2229.
- (31) Hotz, J.; Meier, W. Vesicle-Templated Polymer Hollow Spheres. *Langmuir* **1998**, *14*, 1031–1036.
- (32) Lasic, D. D. Sterically Stabilized Vesicles. *Angew. Chem., Int. Ed.* **1994**, *33*, 1685–1698.
- (33) Gomes, J. F. P. d. S.; Sonnen, A. F. P.; Kronenberger, A.; Fritz, J.; Coelho, M. A. N.; Fournier, D.; Fournier-Nöel, C.; Mauzac, M.; Winterhalter, M. Stable Polymethacrylate Nanocapsules from Ultraviolet Light-Induced Template Radical Polymerization of Unilamellar Liposomes. *Langmuir* **2006**, *22*, 7755–7759.
- (34) Jones, I. W.; Hall, H. K., Jr. Demonstration of a Convergent Approach to UV-Polymerizable Lipids bisDenPC and bisSorbPC. *Tetrahedron Lett.* **2011**, *52*, 3699–3701.
- (35) Liu, S.; Sisson, T. M.; O'Brien, D. F. Synthesis and Polymerization of Heterobifunctional Amphiphiles to Cross-Link Supramolecular Assemblies. *Macromolecules* **2001**, *34*, 465–473.
- (36) Muhandiramlage, T. P.; Cheng, Z.; Roberts, D. L.; Keogh, J. P.; Hall, H. K.; Aspinwall, C. A. Determination of Pore Size and Relative Porosity in Porous Nanoshell Architectures Using Dextran Retention with Single Monomer Resolution and Proton Permeation. *Anal. Chem.* **2012**, *84*, 9754–9761.
- (37) Richter, A. G.; Dergunov, S. A.; Ganus, B.; Thomas, Z.; Pingali, S. V.; Urban, V.; Liu, Y.; Porcar, L.; Pinkhassik, E. Scattering Studies of Hydrophobic Monomers in Liposomal Bilayers: An Expanding Shell Model of Monomer Distribution. *Langmuir* **2011**, *27*, 3792–3797.
- (38) Roberts, D. L.; Ma, Y.; Bowles, S. E.; Janczak, C. M.; Pyun, J.; Saavedra, S. S.; Aspinwall, C. A. Polymer-Stabilized Phospholipid Vesicles with a Controllable, pH-Dependent Disassembly Mechanism. *Langmuir* **2009**, *25*, 1908–1910.
- (39) Okumoto, S.; Jones, A.; Frommer, W. B. Quantitative Imaging with Fluorescent Biosensors. *Annu. Rev. Plant Biol.* **2012**, *63*, 663–706.
- (40) Tamura, T.; Hamachi, I. Recent Progress in Design of Protein-Based Fluorescent Biosensors and Their Cellular Applications. *ACS Chem. Biol.* **2014**, *9*, 2708–2717.
- (41) Ibraheem, A.; Campbell, R. E. Designs and Applications of Fluorescent Protein-Based Biosensors. *Curr. Opin. Chem. Biol.* **2010**, *14*, 30–36.
- (42) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. Creating New Fluorescent Probes for Cell Biology. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906–918.
- (43) Lippincott-Schwartz, J.; Patterson, G. H. Development and Use of Fluorescent Protein Markers in Living Cells. *Science* **2003**, *300*, 87–91.
- (44) Shaner, N. C.; Campbell, R. E.; Steinbach, P. A.; Giepmans, B. N. G.; Palmer, A. E.; Tsien, R. Y. Improved Monomeric Red, Orange and Yellow Fluorescent Proteins Derived from Discosoma Sp. Red Fluorescent Protein. *Nat. Biotechnol.* **2004**, *22*, 1567–1572.
- (45) Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y. A Guide to Choosing Fluorescent Proteins. *Nat. Methods* **2005**, *2*, 905–909.
- (46) Thomas, C. F.; Luisi, P. L. RNA Selectively Interacts with Vesicles Depending on Their Size. *J. Phys. Chem. B* **2005**, *109*, 14544–14550.
- (47) Kolchens, S.; Ramaswami, V.; Birgenheier, J.; Nett, L.; O'Brien, D. F. Quasi-Elastic Light Scattering Determination of the Size Distribution of Extruded Vesicles. *Chem. Phys. Lipids* **1993**, *65*, 1–10.
- (48) Bright, L. K.; Baker, C. A.; Bränström, R.; Saavedra, S. S.; Aspinwall, C. A. Methacrylate Polymer Scaffolding Enhances the Stability of Suspended Lipid Bilayers for Ion Channel Recordings and Biosensor Development. *ACS Biomater. Sci. Eng.* **2015**, *1*, 955–963.
- (49) Ohno, H.; Takeoka, S.; Iwai, H.; Tsuchida, E. Polymerization of Liposomes Composed of Diene-Containing Lipids by Radical Initiators. II*. Polymerization of Monodiene-Type Lipids as Liposomes. *J. Polym. Sci., Part A: Polym. Chem.* **1987**, *25*, 2737–2746.
- (50) Chernomordik, L. V.; Kozlov, M. M. Mechanics of Membrane Fusion. *Nat. Struct. Mol. Biol.* **2008**, *15*, 675–683.
- (51) Enoki, S.; Saeki, K.; Maki, K.; Kuwajima, K. Acid Denaturation and Refolding of Green Fluorescent Protein. *Biochemistry* **2004**, *43*, 14238–14248.
- (52) Stepanenko, O. V.; Verkhusha, V. V.; Kazakov, V. I.; Shavlovsky, M. M.; Kuznetsova, I. M.; Uversky, V. N.; Turoverov,

K. K. Comparative Studies on the Structure and Stability of Fluorescent Proteins EGFP, zFP506, mRFP1, “dimer2”, and DsRED1. *Biochemistry* **2004**, 43, 14913–14923.

(53) Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **1999**, 185, 129–188.

(54) de Lencastre Novaes, L. C.; Mazzola, P. G.; Pessoa, A., Jr.; Penna, T. C. V. Citrate and Phosphate Influence on Green Fluorescent Protein Thermal Stability. *Biotechnol. Prog.* **2010**, 27, 269–272.

(55) Campbell, T. N.; Choy, F. Y. M. The Effect of pH on Green Fluorescent Protein: A Brief Review. *Mol. Biol. Today* **2001**, 2, 1–4.

(56) D'Auria, S.; Ausili, A.; Marabotti, A.; Varriale, A.; Scognamiglio, V.; Staiano, M.; Bertoli, E.; Rossi, M.; Tanfani, F. Binding of Glucose to the D-Galactose/D-Glucose-Binding Protein from *Escherichia coli* Restores the Native Protein Secondary Structure and Thermostability That Are Lost upon Calcium Depletion. *J. Biochem.* **2006**, 139, 213–221.

(57) Kane, R. S.; Stroock, A. D. Nanobiotechnology: Protein-Nanomaterial Interactions. *Biotechnol. Prog.* **2007**, 23, 316–319.

(58) Fukuda, H.; Arai, M.; Kuwajima, K. Folding of Green Fluorescent Protein and the Cycle3 Mutant. *Biochemistry* **2000**, 39, 12025–12032.

(59) Mazzola, P. G.; Ishii, M.; Chau, E.; Cholewa, O.; Penna, T. C. V. Stability of Green Fluorescent Protein (GFP) in Chlorine Solutions of Varying pH. *Biotechnol. Prog.* **2006**, 22, 1702–1707.

(60) Xiao, D. S.; Rong, M. Z.; Zhang, M. Q. A Novel Method for Preparing Epoxy-Containing Microcapsules via UV Irradiation-Induced Interfacial Copolymerization in Emulsions. *Polymer* **2007**, 48, 4765–4776.

(61) Tsuchida, E.; Hasegawa, E.; Kimura, N.; Hatashita, M.; Makino, C. Polymerization of Unsaturated Phospholipids as Large Unilamellar Liposomes at Low Temperature. *Macromolecules* **1992**, 25, 207–212.

(62) Groves, J. T.; Boxer, S. G. Micropattern Formation in Supported Lipid Membranes. *Acc. Chem. Res.* **2002**, 35, 149–157.

(63) Gallagher, E. S.; Adem, S. M.; Baker, C. A.; Ratnayaka, S. N.; Jones, I. W.; Hall, H. K., Jr.; Saavedra, S. S.; Aspinwall, C. A. Highly Stabilized, Polymer-Lipid Membranes Prepared on Silica Microparticles as Stationary Phases for Capillary Chromatography. *J. Chromatogr. A* **2015**, 1385, 28–34.

(64) Miao, Z. -L.; Deng, Y. -J.; Du, H. -Y.; Suo, X. -B.; Wang, X. -Y.; Wang, X.; Wang, L.; Cui, L. -J.; Duan, N. Preparation of a Liposomal Delivery System and Its in Vitro Release of Rapamycin. *Exp. Ther. Med.* **2015**, 9, 941–946.

(65) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 years of Image Analysis. *Nat Methods* **2012**, 9, 671–675.