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

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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 ± 4% vs control), and unaffected by neutral redox initiation (97 ± 3% vs control). Fluorescence emission intensity of encapsulated td-Tomato was significantly attenuated under thermal initiation (13 ± 3% vs control), moderately attenuated UV photoinitiation (64 ± 5% vs control), and unaffected by neutral redox initiation (98% ± 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, nanotechnology, biosensor development, and drug delivery. 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. 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.

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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, including (but not limited to) the use of polymerizable lipids, polymerization of small hydrophobic monomers in the nanoshell bilayer, and surface grafting of water-soluble polymers. 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. 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. This approach eliminates the requirement of polymerizable lipid synthesis, reduces the leakage of encapsulated molecules, 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. Fluorescent protein biosensors have evolved as analytical tools for measurement and/or visualization of specific analytes in vitro and in vivo. 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 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). Following preparation, ethylene glycol dimethacrylate (EGDMA) and n-butyl methacrylate (BMA) monomers were partitioned into the bilayer of the nanoshells and subsequently polymerized (Scheme 1) using three different initiation methods (Irgacure 907; azobis(2-methylpropionamidine) dihydrochloride, AAPD; and ammonium persulfate (NH4)2S2O8/NaHSO3).

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). 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. 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 ~143 ± 3 nm [polydispersity index (PDI) = 0.11], whereas the average diameter of PSS-phospholipid nanoshells was ~300 ± 15 nm (PDI = 0.25), ~480 ± 11 nm (PDI = 0.20), and ~340 ± 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 hydrophobic 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. 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, resulting from the scattering of individual nanoshells, of the phospholipid nanoshell solution at [TX-100]/[DOPC] = 0 (red) and [TX-100]/[DOPC] = 10 (green) was monitored in response to surfactant (TX-100) solubilization at [TX-100]/[DOPC] = 0 (red) and [TX-100]/[DOPC] = 10 (green). The optical density of unstabilized phospholipid nanoshells approaches zero when [TX-100]/[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 ([TX-100]/[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 [TX-100]/[DOPC] = 10. In contrast, upon treatment of PSS-phospholipid nanoshells with excess TX-100 at [TX-100]/[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, which was not detected by the DLS instrument. It should be noted that treatment of PSS-phospholipid nanoshells with excess TX-100 ([TX-100]/[DOPC] = 10) is an extremely harsh condition, which would not be encountered under typical utilization conditions. Therefore, the formation of polymer scaffolds should provide

![Figure 1](ACS Omega 2018, 3, 15890–15899)
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 initiation 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. 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. 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. 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.

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 (%)<sup>a</sup> |
|---------------------------|------------------------------------------|
| initation                 | 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                      |

<sup>a</sup>Relative 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 (%)<sup>a</sup> |
|---------------------------|------------------------------------------|
| initation                 | 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                      |

<sup>a</sup>Relative 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.
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. 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.
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 epi-fluorescence 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 surfaces 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...
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 for 0 h to completely remove trace CHCl₃. Dried lipid films were rehydrated using 1 mL of PBS buffer (pH 7.4). The lipid solution was flushed with Ar, warmed to 42 °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 °C) and warm water (42 °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) (NH₄)₂S₂O₈/NaHSO₃ 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 °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 h. Monomer-doped nanoshell was added and stirred in the dark to complete polymerization. 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 °C in the dark for 17 h with continuous stirring to initiate polymerization.

Neutral Redox Initiation. (NH₄)₂S₂O₈ and NaHSO₃ are used as neutral redox initiator pair. 63 (NH₄)₂S₂O₈ and NaHSO₃ were dissolved separately in PBS buffer at 10 mg/mL, each immediately before use. (NH₄)₂S₂O₈ and NaHSO₃ 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.
°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 H_2O and freeze-dried. Because methanol is used to break the phospholipid nanoshells 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. 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 backscattering 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 JEOL NABITY Nanometer Pattern Generation System. Protein-encapsulated PSS-phospholipid nanoshells were imaged using a Hamamatsu Imagent X2 digital camera attached to a Nikon Eclipse TE300 Quantum inverted epifluorescence microscope. Emission was collected through an oil-immersion objective (40X, numerical aperture = 1.30), with a filter cube (green filter, 540 nm/25 nm; red filter, 620 nm/60 nm). HClImage Live software was used to collect the images, and ImageJ software was used to analyze the images.

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