Permeable Protein-Loaded Polymersome Cascade Nanoreactors by Polymerization-Induced Self-Assembly

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ABSTRACT: Enzyme loading of polymersomes requires permeability to enable them to interact with the external environment, typically requiring addition of complex functionality to enable porosity. Herein, we describe a synthetic route towards intrinsically permeable polymersomes loaded with functional proteins using initiator-free visible light-mediated polymerization-induced self-assembly (photo-PISA) under mild, aqueous conditions using a commercial monomer. Compartmentalization and retention of protein functionality was demonstrated using green fluorescent protein as a macromolecular chromophore. Catalytic enzyme-loaded vesicles using horseradish peroxidase and glucose oxidase were also prepared and the permeability of the membrane towards their small molecule substrates was revealed for the first time. Finally, the interaction of the compartmentalized enzymes between separate vesicles was validated by means of an enzymatic cascade reaction. These findings have a broad scope as the methodology could be applied for the encapsulation of a large range of macromolecules for advancements in the fields of nanotechnology, biomimicry and nanomedicine.

Compartmentalization is essential for all forms of life. For instance, cells and organelles can interact with one another through enzyme cascades, and through transport of signalling molecules, energy and nutrients. Mimicry of these natural constructs using synthetic materials is of fundamental scientific interest, and could also lead to advances in bionanotechnology and nanomedicine.[1-3] Self-assembled bilayer structures, such as liposomes, used to encapsulate functional macromolecules, can be considered as minimal artificial cells, or protocells.[2,4] Amphiphilic block copolymer vesicles (also termed polymersomes) have been studied widely as such protocells, owing to their higher mechanical strength and easier functionalization when compared to liposomes. For example, Lecommondoux and van Hest *et al*. demonstrated that poly(styrene)-*b*-poly(3-(isocyano-L-alanyl-aminoethyl)thiophene)) (PS-*b*-PIAT) vesicles loaded with enzymes could be encapsulated inside a larger poly(butadiene)-*b*-poly(ethylene glycol) (PB-*b*-PEG) polymersome to create a multi-compartmentalized polymersome-in-polymersome system, which structurally resembled a cell and its organelles.[5] The encapsulated enzymes were able to interact *via* an enzymatic cascade reaction. Reactants and products were able to diffuse between the enzyme-containing compartments owing to the intrinsic permeability of these PS-*b*-PIAT polymersomes, comprising specialty monomers and polymerization techniques. Whilst examples of enzyme-loaded polymersome nanoreactors are numerous,[1-14] examples of membrane-forming polymers with intrinsic permeability are rarely reported. As such, the non-permeable nature of the membrane must be overcome by the incorporation of membrane proteins[13,15,16] or DNA nanopores[17] into the polymersome membrane, post-assembly radical photoreactions,[12] or the use of stimuli-responsive membranes[14,18-20] to impart permeability. Furthermore, the preparation of block copolymer vesicles often requires the use of organic solvents, which may be incompatible with the protein of interest. Additionally, conventional self-assembly procedures are typically performed at low concentrations, and require multiple synthetic and purification steps, which limits their scalability.

Herein, we report the intrinsic permeability of poly(ethylene glycol)-*b*-poly(2-hydroxypropyl methacrylate) (PEG-*b*-PHPMA) vesicles formed by a one-step aqueous, initiator-free, visible light-mediated polymerization-induced self-assembly (photo-PISA) route using commercial reagents (Fig. 1A). This approach overcomes many of the challenges discussed for conventional protein-loaded block copolymer vesicles, such as their lack of intrinsic permeability. PISA shows numerous advantages over other self-assembly techniques, such as the use of purely aqueous conditions and high concentrations (in this case, 110 mg·mL^{-1}).[21-31] This single, rapid assembly methodology could feasably be applied to the encapsulation of a range of functional proteins. Bovine serum albumin (BSA), a robust non-functional protein, has been the main focus of protein encapsulation inside PISA-derived vesicles.[26,32] In more recent work, BSA-functionalized PISA-derived nanoparticles were prepared by direct polymerization of HPMA from the chain transfer agent (CTA)-functionalized protein.[33] Very recently, Tan and Zhang *et*

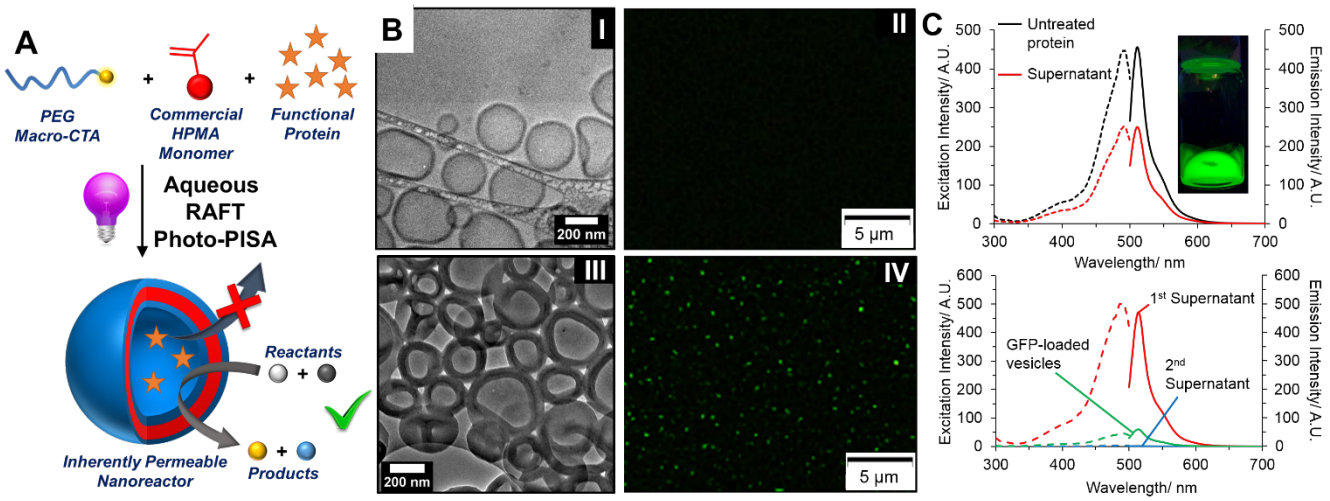

Fig. 1. A: Preparation of inherently permeable protein-loaded nanoreactors by aqueous PISA. B: Cryo-TEM image and fluorescence micrograph of empty vesicles (I and II) and GFP-loaded vesicles (III and IV). C (Top): Fluorescence spectra of the 1st supernatant from the purification (red) and the untreated protein (black). The inset shows a digital photograph of the crude reaction mixture under a UV lamp. (Bottom): Fluorescence spectra of the 1st supernatant (red), disassembled vesicle solution (green) and 2nd supernatant (blue) in a 5:1 methanol : water solvent mixture. In each case the excitation spectra (λ_{Em} = 512 nm, dashed traces) and the emission spectra (λ_{Ex} = 495 nm, solid traces) are shown.

al. described enzyme-assisted photoinitiated PISA using glucose oxidase to degas the solution by reduction of dissolved oxygen.[34] Whilst this achieved glucose oxidase- and horseradish peroxidase-loaded vesicles by PISA, and whilst they demonstrated no loss in native (unencapsulated) activity, the permeable nature of the PHPMA membrane was not investigated and so the authors were unable to show that the enzyme remained active whilst encapsulated inside the vesicle.

Polymer synthesis and preparation of GFP-loaded vesicles. Aqueous photo-PISA without the need for an initiator was employed to prepare polymersomes comprising a PEG shell and a PHPMA membrane (Fig. 1A). Such membranes are highly hydrated,[22] which was hypothesized to allow size-selective transport of small molecules whilst retaining the encapsulated protein. From our previous work on photoinitiated PISA,[35] a PEG_{113}-*b*-$PHPMA_{400}$ block copolymer formed at 10 wt% HPMA resulted in the reliable preparation of unilamellar vesicles when photoinitiated PISA was employed at 37 °C. This point on the phase diagram was used for the preparation of all protein-loaded vesicles discussed. Size exclusion chromatography (SEC) of each of the polymers confirmed that the diblock copolymer synthesis was not strongly affected by the presence of the encapsulated proteins in the reaction mixture (Fig. S1). The empty vesicles were characterized by dry state stained transmission electron microscopy (TEM), cryogenic TEM (cryo-TEM) and dynamic light scattering (DLS) analysis (Fig. S2). Cryo-TEM confirmed the vesicles' unilamellar character (Fig. 1B-I), and statistical analysis from 200 particles revealed an average membrane thickness of 25 ± 4 nm.

In order to confirm compartmentalization in our system, encapsulation of a recombinant enhanced green fluorescent protein (GFP) was achieved by dissolution of the PEG_{113} macro CTA and HPMA monomer in an aqueous solution of GFP followed by irradiation at 400-410 nm at 37 °C to initiate the PISA. The GFP-loaded vesicles were purified from the unencapsulated protein by three centrifugation-resuspension cycles at relatively low centrifugal forces (16,000 × g for 10 min) such that the unencapsulated protein remained in the supernatant after each cycle. The vesicles were fully characterized after purification (Fig. S3 and Fig. 1B-III). The average membrane thickness of the vesicles was determined by cryo-TEM to be 52 ± 12 nm. This membrane thickness was significantly higher than that of the empty vesicles (25 ± 4 nm). This was attributed to the presence of residual salts present following the protein purification, which have an effect on the PISA assembly process, as will be discussed later when glucose oxidase (GOx)-loaded vesicles are explored. Fluorescence spectroscopy of the first supernatant from the purification revealed a loss in fluorescence after the PISA process relative to fresh GFP, which was attributed in part to photobleaching during the light-mediated polymerization,[36] however 56% of the protein's fluorescence was retained (Fig. 1C, top). Additionally, the purified vesicles' fluorescence could be assessed in order to calculate the loading efficiency of the protein. A loading efficiency of 11 ± 2%, was calculated upon disassembly of the vesicles followed by fluorescence spectroscopy (Fig. 1C, bottom). Fig. 1B shows a fluorescence micrograph of the GFP-loaded vesicles, which demonstrated that the fluorescent protein was compartmentalized into discreet pockets inside the vesicles' lumens. Control experiments wherein empty PISA vesicles containing no GFP were incubated with GFP and irradiated with light under the same conditions, followed by an identical purification procedure, revealed no fluorescent features (Fig. S4), demonstrating that the fluorescence observed was from encapsulated protein and not

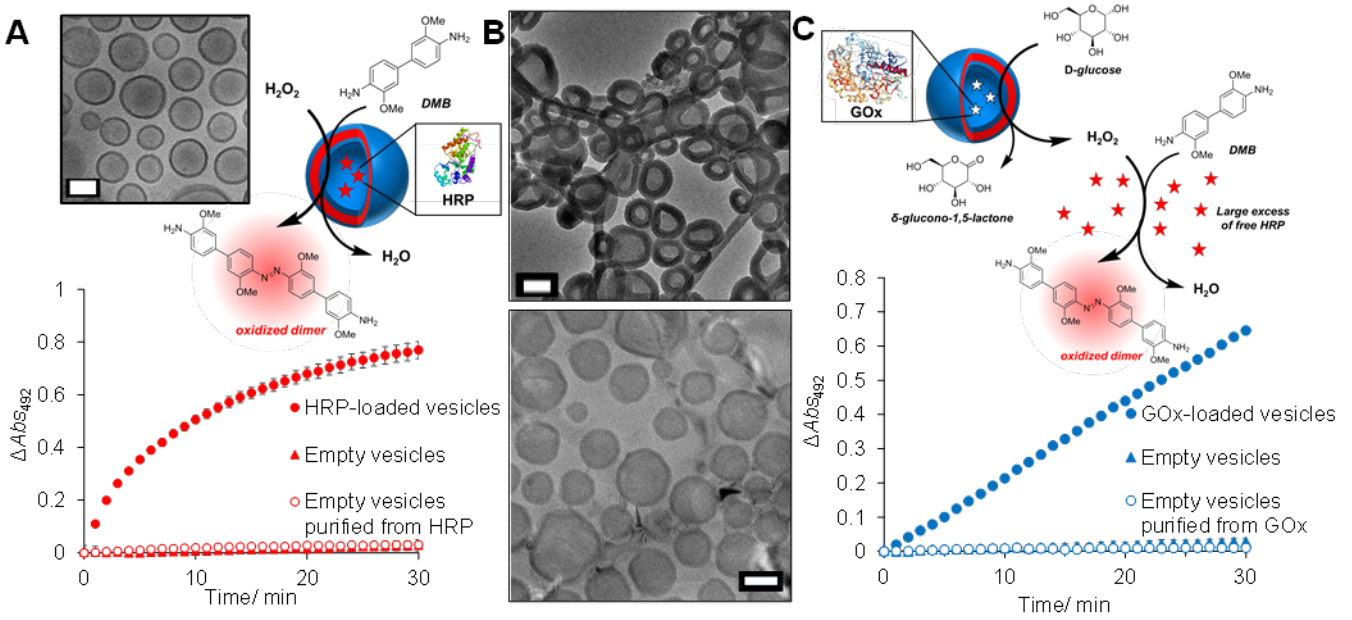

Fig. 2. A (Inset): Cryo-TEM image of HRP-loaded vesicles. (Top): Schematic showing the HRP-catalyzed oxidation of DMB to its colored dimer product detected in the colorimetric assay. (Bottom): Activity of the HRP-loaded vesicles against negative controls. B: Cryo-TEM images of GOx-loaded vesicles using commercial GOx (top) and purified GOx (bottom). C (Top): Schematic showing the GOx-catalyzed oxidation of D-glucose to δ-glucono-1,5-lactone. Excess HRP in the external solution was then used to catalyze the oxidation of DMB using hydrogen peroxide product from the first step. (Bottom): Activity of the GOx-loaded vesicles against negative controls. The error bars in A and C show the standard deviation from 4 repeats. All scale bars = 200 nm.

from protein adhered to the particle's surface. Whilst this was not the first report of GFP-loaded PISA-derived vesicles,[37] these initial results were used as a basis for studying our PEG_{113}-$PHPMA_{400}$ system, rather than a polymer-protein conjugate PISA vesicle system used in the aforementioned report.

Catalytic HRP-loaded nanoreactors. Horseradish peroxidase (HRP) was used as a model enzyme for encapsulation to prepare PISA-derived vesicles with a catalytic function. These HRP- loaded vesicles were prepared using a similar method to that used for the GFP-loaded vesicles and gave a broad, unimodal distribution of particle sizes as characterized by DLS, dry state TEM and cryo-TEM (Fig. S5 and Fig. 2A). Again, the particles survived centrifugation and were also stable to purification by preparative SEC to ensure the complete removal of unencapsulated protein species. The activity of the HRP loaded inside the vesicle was assessed using a colorimetric assay, in which HRP was used to catalyze the oxidation of 3,3'-dimethoxybenzidine (DMB) to a colored dimer product (Fig. 2A). It was found that the enzyme's activity was retained despite its compartmentalization from the external medium. This demonstrated the permeability of the moderately hydrophobic PHPMA membrane-forming block towards DMB and hydrogen peroxide. To ensure complete removal of the free protein, the purification methodology was interrogated and several control experiments were performed. It was demonstrated that the proteins were not adhering to the particles' surfaces by incubating the free protein with empty vesicles with light irradiation. Following an identical purification procedure, the activity of this control was shown to be negligible (Fig. 2A). The preparative SEC traces of the HRP-loaded vesicles and of the free, unencapsulated HRP also revealed that the retention time of the very high molar mass vesicles was much lower than that of the free protein, such that the fractions collected for assessment of the vesicles' activity did not contain any free enzyme in solution (Fig. S6). These control experiments demonstrated that the observed activity in the HRP-loaded vesicles was solely a result of encapsulated protein and not that of protein adhered physically or chemically to the vesicle surface. Western blot analysis of the disassembled vesicles revealed a loading efficiency of 27.5%. This efficiency could also be increased to 54% by increasing the initial protein feed five-fold. The encapsulated enzyme retained 57 ± 13% of its activity relative to the free enzyme (Fig S7). The loss in activity could possibly be attributed to the diffusive barrier of the membrane, which hampers the substrates in reaching the active site of the enzyme.

Preparation of GOx-loaded vesicles and cascade activity. To expand the scope of enzyme-loaded PISA vesicles, those loaded with GOx were prepared as this protein has been shown to be able to interact with HRP by means of a catalytic cascade reaction.[8,14] GOx-loaded vesicles were prepared in a similar manner to HRP- and GFP-loaded vesicles and the purified particles were fully characterized (Fig. S8 and Fig. 2B). Similar to the GFP-loaded vesicles, cryo-TEM of the GOx-loaded vesicles showed significantly thicker membranes than the empty vesicles or the HRP-loaded vesicles. This was attributed to the purity of the commercially available GOx, which is sup-

plied as a lyophilized powder with salts and other stabilizing agents. Vesicles loaded with a purified, desalted GOx had an identical membrane thickness to the empty vesicles, within error, indicating that the protein itself did not have an effect on the PISA assembly process (Fig. 2B). GOx catalyzes the oxidation of D-glucose to δ-glucono-1,5-lactone and produces hydrogen peroxide as a byproduct. Since this reaction gives no colorimetric output, a large excess of fresh, unencapsulated HRP was employed, along with DMB, in order to facilitate the oxidation of DMB to its colored dimer product using hydrogen peroxide as a rate limiting substrate (Fig. 2C). In this way, the activity of the GOx could be measured. Similar to HRP-loaded vesicles, the GOx-loaded vesicles also showed catalytic activity, whereas the empty vesicles, and the empty vesicles purified from a GOx-containing solution after light irradiation, showed no appreciable activity (Fig. 2C). Similarly to HRP, Western blot analysis of the disassembled vesicles revealed a loading efficiency of 24%, and again this could be increased to 41% by increasing the initial protein feed five-fold. Relative to the free enzyme, the encapsulated enzyme retained 46 ± 1% of its activity, owing to the diffusive barrier imparted by the membrane (Fig S9).

Once it had been shown that substrates for both GOx and HRP could permeate through the polymersome membrane, it was possible to assess if these two enzymes, separated in space by compartmentalization of their vesicle lumens, could interact with one another by utilization of a cascade reaction. HRP- and GOx-loaded vesicles were prepared in separate PISA formulations, purified as described previously and mixed in a 1:1 ratio in phosphate buffer. DMB was added as a reporter to give a colorimetric output, followed by D-glucose to initiate the cascade. Fig. 3 shows that the cascade proceeded only when all components were present in solution. Controls where just one species was present, or when D-glucose was absent, showed no activity. This further demonstrated the permeability towards small molecules owing to the hydrated nature of PHPMA, allowing the crossing of substrates and products both in and out of the vesicle membrane. Relative to the free enzymes, the cascade proceeded with 46 ± 3% activity, owing to the hindered passage of substrates through the membrane (Fig. S10).

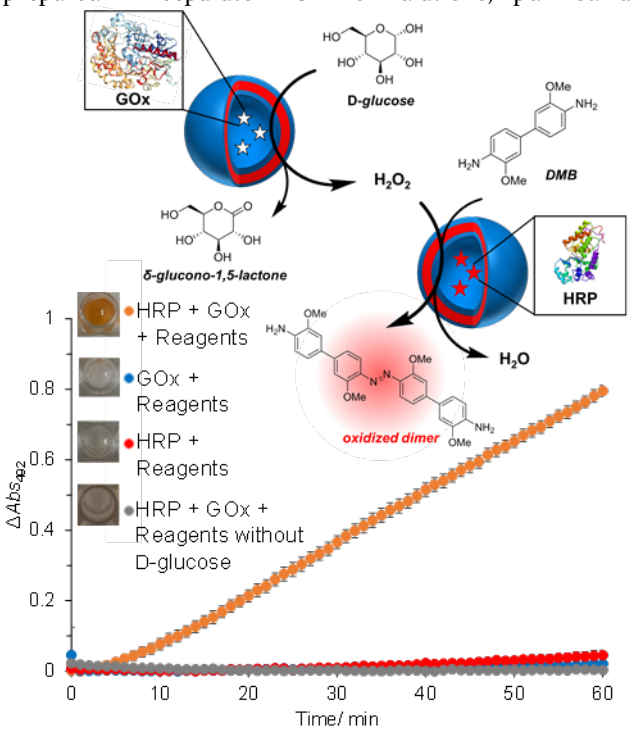

Fig. 3. Schematic and activity of the cascade reaction between HRP- and GOx-loaded vesicles with negative controls. Error bars show the standard deviation from 4 repeats. Insets: End point photographs of wells after 1.5 h.

We report for the first time the intrinsically permeable nature of PHPMA membranes towards a range of small molecule substrates. Functional proteins could be retained within compartmentalized domains and preserve their function, as revealed by fluorescence microscopy of GFP-loaded vesicles. Enzymes were also found to remain active, owing to the intrinsic permeability of the membrane, such that the compartmentalized enzymes could interact by way of a cascade reaction. This one-pot, mild approach is highly versatile and could be applied to a range of functional enzymes. As PHPMA is one of the most reported monomers for aqueous PISA found in the literature, it is expected that this phenomenon will be widely exploited for a range of bio-related applications.

ASSOCIATED CONTENT

Particle characterization, SEC traces including preparative SEC traces of the HRP-loaded vesicles, calculation of loading efficiencies and GFP control images are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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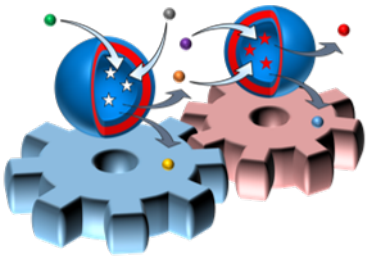

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