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Polymer Vesicle Microreactors Produced using Permeable Polymer Blocks: Circumventing Complex Functionality to Impart Membrane Permeability

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ABSTRACT

The use of giant vesicles as microreactors presents a novel approach to control biochemical reactions in confined spaces, offering advantages such as compartmentalization, tunable permeability, and potential for biomimetic applications. These constructs can serve as versatile platforms for catalysis, drug delivery, and synthetic biology by providing confined environments that mimic natural cellular compartments. We have successfully produced microvesicles (also referred to as giant vesicles) by means of the simple double emulsification method using five amphiphilic block copolymers comprising poly(ethylene oxide) (PEO) as hydrophilic segment and five disparate hydrophobic blocks: poly(caprolactone) (PCL), poly(methyl methacrylate) (PMMA), poly(lactic acid) (PLA), poly[2-(diisopropylamino)ethyl methacrylate] (PDPA), and poly[2-(heptamethyleneimino)ethyl methacrylate] (PHIA). The last two blocks are pH-responsive (PDPA, PHIA), while the first ones are not (PCL, PMMA, PLA). The resulting vesicles have average size ranging from 2.9 to 9.3 μm , with the pH-responsive vesicles exhibiting larger diameters, likely due to partial protonation of the hydrophobic blocks. The formation of the giant vesicles was confirmed *via* optical and fluorescence microscopy using Nile red as a hydrophobic marker. The ability of the vesicles to encapsulate larger molecules was demonstrated by loading Alexa-labeled bovine serum albumin (BSA-Alexa). In the step further, the potential of these vesicles as microreactors was explored by encapsulating horseradish peroxidase enzyme (HRP) and evaluating the catalytic oxidation of o-dianisidine in the presence of hydrogen peroxide (H_2O_2), a reaction catalyzed by the HRP enzyme. The experimental evidences highlight that the pH-responsive vesicles are permeable to the reactants, as evidenced by colored product formation, whereas the permeability of the nonresponsive assemblies is reported to be negligible. Truly, the non-responsive vesicles exhibited particularly low

permeability, even at the pH where the catalytic activity of the enzyme is optimized. These findings highlight the potential of pH-responsive vesicles for controlled molecular transport and catalytic applications, paving the way for their use in biocatalysis as microreactors.

Keywords: self-assembly, block copolymers, giant polymer vesicles, microreactors

INTRODUCTION

Compartmentalization is a core principle in cell construction, and essential for orchestrating the precision of biological processes.¹ The ability of nature to create such functional and dynamic outstanding superstructure has motivated the mimicking and the design of compartments whose membranes are formed using various amphiphilic molecules. In such approaches, synthetic vesicles respectively holding an aqueous lumen are shaped and the confined spaces are able to regulate chemical and biochemical reactions, while simultaneously shielding delicate components (such as sensitive enzymes) from external detrimental agents.^{2,3} The compartmentalized environments can be size-tuned with size ranging from few nanometers up to roughly 20 μm depending on the methodology used in the bottom-up process.⁴⁻⁹ Accordingly, these constructs receive great attention in biotechnology and they are frequently considered in the production of synthetic cells, and on the manufacturing of micro and nanoreactors for biocatalysis.^{3,10-}

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The polymer vesicles (also named polymersomes), which are produced by the self-assembly of amphiphilic block copolymers, are indeed significantly more stable than the more classical lipid vesicles (named liposomes), which are produced by the self-assembly of phospholipids. The lower stability of liposomes is mainly the consequence of the high lateral fluidity linked to the low molecular weight of lipids.¹⁵ On the other hand, the higher molecular weight of polymers typically results in thicker membranes, reduced lateral fluidity and low polymersome permeability, although this property depends on the physicochemical features of the hydrophobic segment.¹⁶ While low membrane permeability is desired to retain cargos with greater efficiency, the more restricted communication between the inner and outer compartments, and the limited

capability to regulate the flow of chemicals in polymersomes is problematic in certain applications (e.g. enzymatic micro and nanoreactors).¹⁷ In this framework, the insertion of membrane proteins into the polymeric membranes is certainly the most common approach to bypass reduced polymer vesicle permeability. This has been evidenced by using various channel proteins such as outer membrane porin OmpF and phosphoporin PhoE¹⁸, staphylococcal α -hemolysin (α HL)¹⁹ and the bacterial water-channel protein Aquaporin Z (AqpZ)²⁰, for instance. Using this concept, a variety of enzymes including penicillin acylase,²¹ horseradish peroxidase²² and β -lactamase²³ (to name a few) have been encapsulated into disparate hybrid vesicles where substrates and products could be transported through the walls while the macromolecules remained entrapped. Nevertheless, although the insertion of biopores (membrane proteins) can be achieved spontaneously during manufacturing of the polymer vesicles, the process is governed by insertion kinetics which is commonly difficult to control.²⁴ Additionally, effective protein insertion depends on its dimension that has to some extent to fit with the thickness of the polymeric membrane, which is typically not the case. Accordingly, the production of permeable and semipermeable polymeric membranes without the use of external agents, though still relatively underexplored, represents a significant advancement in the field. Successful strategies to impart polymer vesicle permeability, apart from the insertion of membrane proteins, includes, for instance, the blending of amphiphilic and stimuli-responsive block copolymers where phase-separated domains could be produced and further dissolved by changing the environmental pH,²⁵ or by the presence of reactive oxygen species (H_2O_2).²⁶ Environmentally-responsive polymers can also be used to produce bioreactors without transmembrane proteins, where reversible swelling and deswelling, and respective changes in polymersome permeability, are controlled upon changes in the pH²⁷ when using pH-responsive polymers, or in the temperature²⁸ when

polymer vesicles are manufactured using polymers exhibiting LCST-like behavior (LCST stands for lower critical solution temperature). On the other hand, intrinsically permeable polymer vesicles, regardless environmental conditions, are hardly found. This has been evidenced using rod-coil diblock copolymers such as polystyrene-*b*-poly(L-isocyanoalanine(2-thiophen-3-ylethyl)amide)²⁹ resulting in porous permeable membranes due to frustrated packing. Intrinsically permeable polymer vesicles were also produced using poly(ethylene glycol)-*b*-poly(2-hydroxypropyl methacrylate) due to the hydrated nature of the only moderated hydrophobic poly(2-hydroxypropyl methacrylate) membrane-forming block.³⁰ Truly, the balance of hydrophilicity and hydrophobicity of the polymeric membrane seems to be decisive, and indeed governs the permeability of polymersomes, as chemical reaction of various highly hydrophobic polymers with hydrophilic 2-hydroxy-4'-2-(hydroxyethoxy)-2-methylpropiophenone (PP-OH) increases the permeability of the membranes.³¹ Such membrane features thus allow the manufacturing of micro and nanoreactors without the need of insertional nanopores.

Taking into account all the above-mentioned considerations, we herein demonstrate the manufacturing of intrinsically permeable giant polymer vesicles using amino-containing responsive polymers in the building units. The investigation mainly relies on the block copolymers comprising poly[2-(diisopropylamino)ethyl methacrylate] (PDPA) and poly[2-(heptamethyleneimino)ethyl methacrylate] (PHIA) as hydrophobic segment. These are pH-responsive polymers with $pK_a < 7.4$, where they still preserve a certain degree of protonation at physiological conditions, leading to the formation of hydrate layers, allowing the flow of small organic molecules, while enzymes are too large to cross the membranes. The self-assemblies were produced by a simple double emulsification technique³² enabling high encapsulation efficiency of macromolecules. Giant polymersomes are indeed desired compared to small vesicles of ca. 100 nm

diameters for robust and high-yield formation and enzyme encapsulation.^{32,33} Microreactors were further developed by entrapping homogeneous catalysts in the hollow spheres, and a much faster flow of substrates and products, compared to microvesicles produced using more hydrophobic nonresponsive polymers, has been evidenced. The simple double emulsification technique is, therefore, highlighted as a highly useful tool to produce intrinsically permeable microreactors based on moderately hydrophobic, amino-containing pH-responsive polymers. This is an actual step forward to the field, since the manufacturing of intrinsically permeable polymeric microreactors have been rarely investigated, to be best of our knowledge, and the proposed methodologies circumvent various challenges in producing enzyme-loaded polymer vesicles (microreactors), including primarily the lack to membrane permeability. This simple and rapid assembly and loading methodology could feasibly be applied towards the manufacturing of nanoreactors for a wide array of functional proteins, aiming at the production of various biologically relevant compounds without the need of complex or costly permeabilization methods.

MATERIALS AND CHEMICALS

Chemicals

Horseradish peroxidase (HRP, Type II, lyophilized powder, 150-250 units per mg), o-dianisidine, toluene (99.9% HPLC), chloroform, dibasic sodium phosphate, D-(+)-glucose (Z99.5% GC), 4,4'-azobis (4-cyanovaleric acid) (V501), 2-(diisopropylamino)ethyl methacrylate (97%), heptamethyleneimine (98%), 2-bromoethanol (95%) and hydrogen peroxide 10 % volume were purchased from Sigma-Aldrich. Albumin from bovine serum, Alexa Fluor™ 647 conjugate (BSA-Alexa) was purchased from Invitrogen. Sucrose and acetic acid were respectively purchased from Dinamica Quimica and Synth Lab.

Block Copolymers

The block copolymers poly(ethylene oxide)₁₁₂-*b*-poly(caprolactone)₈₇, poly(ethylene oxide)₇₉-*b*-poly(methyl meth acrylate)₁₉₃, poly(ethylene oxide)₄₅-*b*-poly(lactic acid)₁₇₄ were purchased from Polymer Source.™ Inc. The macro chain-transfer agent PEG macro-CTA poly(ethylene glycol) 4-cyano-4-(phenylcarbonothioylthio)pentanoate (PEG mCTA, $M_n \sim 5\ 000\ \text{g}\cdot\text{mol}^{-1}$) was synthesized according to a previously published investigation³⁴. The pH-responsive block copolymers poly(ethylene oxide)₁₁₃-*b*-poly[2-(diisopropylamino)ethyl methacrylate]₁₈₀ (PEO₁₁₃-*b*-PDPA₁₈₅) and poly(ethylene oxide)₁₁₃-*b*-poly[2-(heptamethyleneimino)ethyl methacrylate]₆₇ (PEO₁₁₃-*b*-PHIA₅₅) were synthesized by us with the synthetic approaches and polymer characterization described in details in the Supporting Information File.

Preparation of the Giant Polymersomes

The giant polymersomes were prepared using a double emulsification method where 20 mg.mL⁻¹ solutions of each copolymer were prepared in toluene or toluene:chloroform (30:70 v/v) mixture according to the solubility of each copolymer. When Nile Red was used to stain the polymer membrane, the dye was added directly to the organic solutions to a final concentration of 10 µg.mL⁻¹. After complete dissolution, 5 µL of an aqueous sucrose solution (250 mM) was mixed with 30 µL of the polymer solutions in a 1.5 mL microcentrifuge tube. This was followed by emulsification for 1 min using a micropipette until the formation of the water-in-oil (W/O) emulsions. The double emulsion droplets were then formed by adding 1000 µL of the aqueous sucrose solution into the W/O emulsions followed by another 1 min gentle emulsification step. The microcentrifuge tubes containing the emulsification was left open to air for at least 3 hours to remove the organic solvents.

Protein Encapsulation into the Giant Polymer Vesicles

The labelled protein (BSA-Alexa) and HRP enzyme were firstly dissolved into the initial sucrose solution used in the first emulsification step of the double emulsification method described above. The vesicles loaded with the HRP enzyme (named HRP-loaded vesicles) were dialyzed against sucrose solution (250 mM) using a regenerated cellulose membrane (MWCO 100 kDa, Spectrum Laboratories, Inc.). The dialysis reservoir was changed twice a day until all unencapsulated HRP was completely removed. This has been conducted with a simultaneous control experiment where free enzyme with the same initial enzyme concentration has been dialyzed and at the end of the purification process, the absence of enzyme was confirmed by using the Bradford method. In the step further, the vesicles were mechanically disrupted by sonication and

pipetting, then concentrated using Amicon Ultra-4 centrifugal filter units. The final concentration of HRP in each sample was determined by UV-Vis spectroscopy using a Nanodrop 2000c spectrophotometer (Thermo Scientific) and the encapsulation efficiency (EE %) of each system was calculated using the following Equation 1.

$$EE\% = \frac{W_t}{W_i} \times 100\% \quad (1)$$

where W_t is the total amount of encapsulated HRP, and W_i is the HRP feeding.

Characterization of the Giant Polymersomes

The giant polymersomes were observed using an optical microscope (Leica DM 2700 M) where 100 μ L of the samples were transferred to a microscope chamber slide containing 400 μ L of aqueous glucose solution (250 mM) for further analysis on a widefield Leica DMI 6000 B microscope (Leica Micro systems, Germany) coupled to an ultrafast Leica DFC365 FX digital camera (Leica Microsystems, Germany). Fluorescence imaging was conducted using excitation filter 525-565 nm and emission filter 572-648 nm which were optimized to detect Nile red and BSA-Alexa fluorophores. The size of at least 300 particles was measured by using the ImageJ software and then the diameters were computed using OriginPro 9.0 for the statistical analysis of average size and size distribution histogram.

Enzymatic Reaction Assays

The enzymatic reactions were carried out on 3 mL UV-Vis cuvettes where 200 μ L of 10% volume H_2O_2 , 500 μ L of either 200 mM acetate (pH 5.5) or phosphate (pH 8.0) buffer, the appropriate volume of HRP-vesicles or free HRP solution to reach a final

enzyme concentration of $1 \mu\text{g}\cdot\text{mL}^{-1}$, and the required amount of sucrose solution to reach a final volume of 1 mL were added. The baselines were recorded with these mixtures before adding 30 μL of a 2.0 mM o-dianisidine solution. The absorption spectrum of the samples was immediately measured over a wavelength ranging from 370 to 550 nm at 30-second interval for the pH-sensitive copolymers and every 2 minutes for the nonresponsive counterparts. The same procedure was carried out using a free enzyme solution at the same concentrations ($1 \mu\text{g}\cdot\text{mL}^{-1}$ at pH 8.0 and 5.5) for comparison purposes. Profiles of maximum absorption as a function of time was generated for each giant polymersome and for the free enzyme and respectively discussed.

RESULTS AND DISCUSSION

The molecular structures of the block copolymers used to produce the self-assemblies at the microscale are provided in Fig. 1.

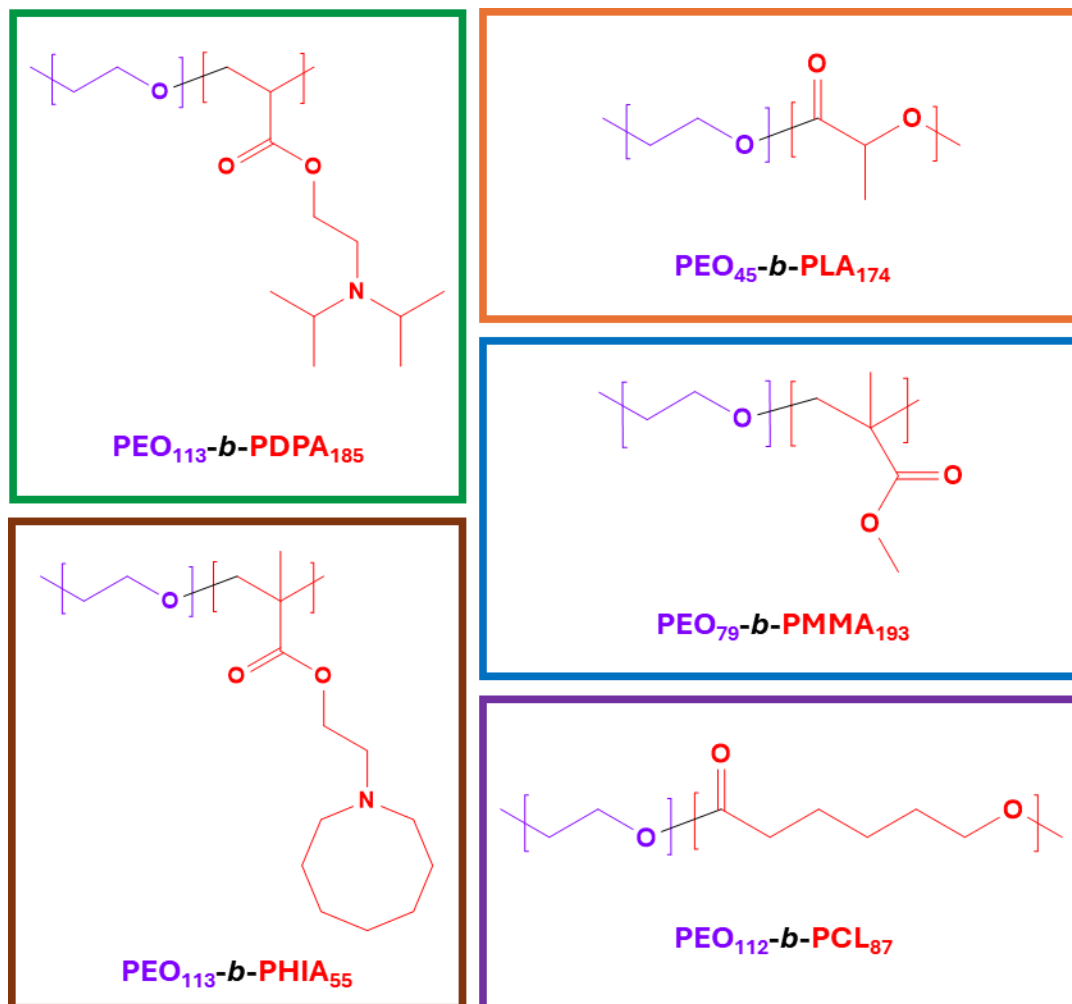


Figure 1. Molecular structure of the block copolymers PEO₄₅-*b*-PLA₁₇₄, PEO₁₁₂-*b*-PCL₈₇, PEO₇₉-*b*-PMMA₁₉₃, PEO₁₁₃-*b*-PDPA₁₈₅ and PEO₁₁₃-*b*-PHIA₅₅ according to the labels.

The pH-responsive block copolymers were synthesized by us and details for monomer and polymer synthesis, as well as polymer characterization are complemented in the Supporting Information File (Figures S1, S2). The composition of the pH-

responsive block copolymers was probed by ^1H NMR spectroscopy. The ^1H NMR spectra of these diblock copolymers ($\text{PEO}_{113}\text{-}b\text{-PHIA}_{55}$, Fig. 2; $\text{PEO}_{113}\text{-}b\text{-PDPA}_{185}$, Fig. 3) underlines the characteristic signals for the protons belonging to PEO, PDPA and PHIA repeating units. The subscripts throughout the manuscript refer to the degrees of polymerization of each block as determined by the polymerization conversions (26 % - $\text{PEO}_{113}\text{-}b\text{-PHIA}_{55}$; 93% - $\text{PEO}_{113}\text{-}b\text{-PDPA}_{185}$ - Supporting Information File, Table S1) given the number-average molecular weight of $M_n = 17\,286\text{ g}\cdot\text{mol}^{-1}$ for $\text{PEO}_{113}\text{-}b\text{-PHIA}_{55}$ and $M_n = 44\,674\text{ g}\cdot\text{mol}^{-1}$ for $\text{PEO}_{113}\text{-}b\text{-PDPA}_{185}$.

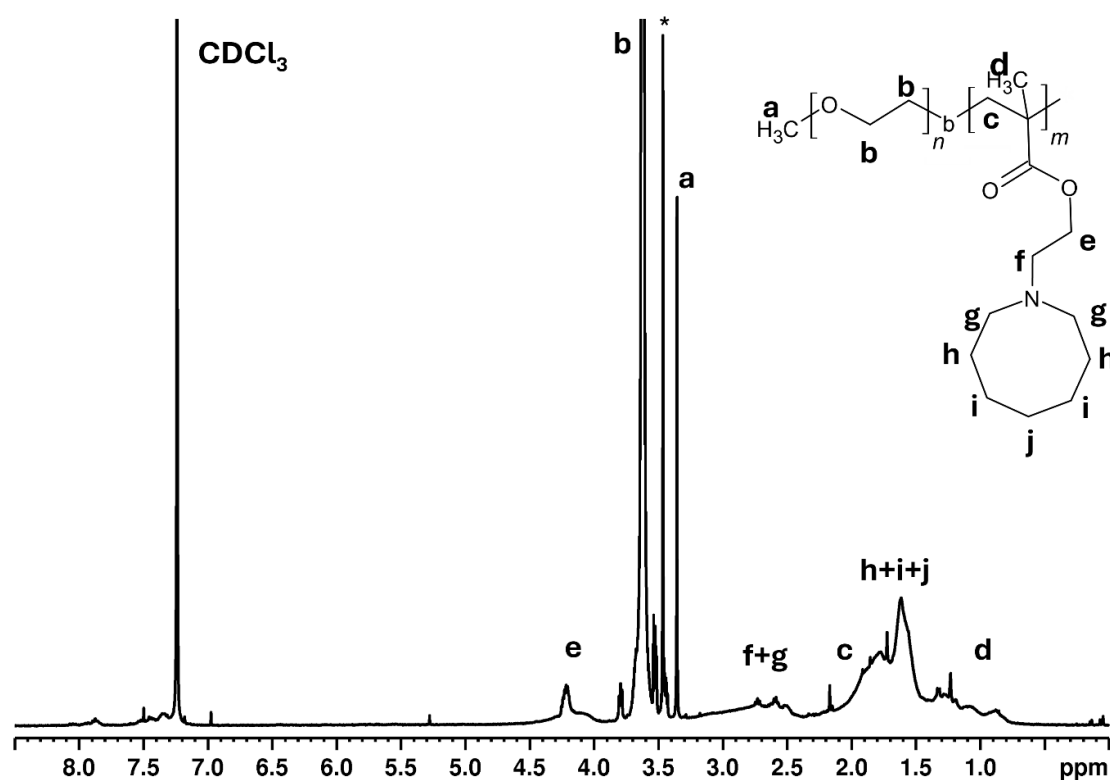


Figure 2. ^1H NMR spectrum for polyethylene oxide-*b*-poly[2-(heptamethyleneimino)ethyl methacrylate] ($\text{PEO}_{113}\text{-}b\text{-PHIA}_{55}$) measured in CDCl_3 at 298K.

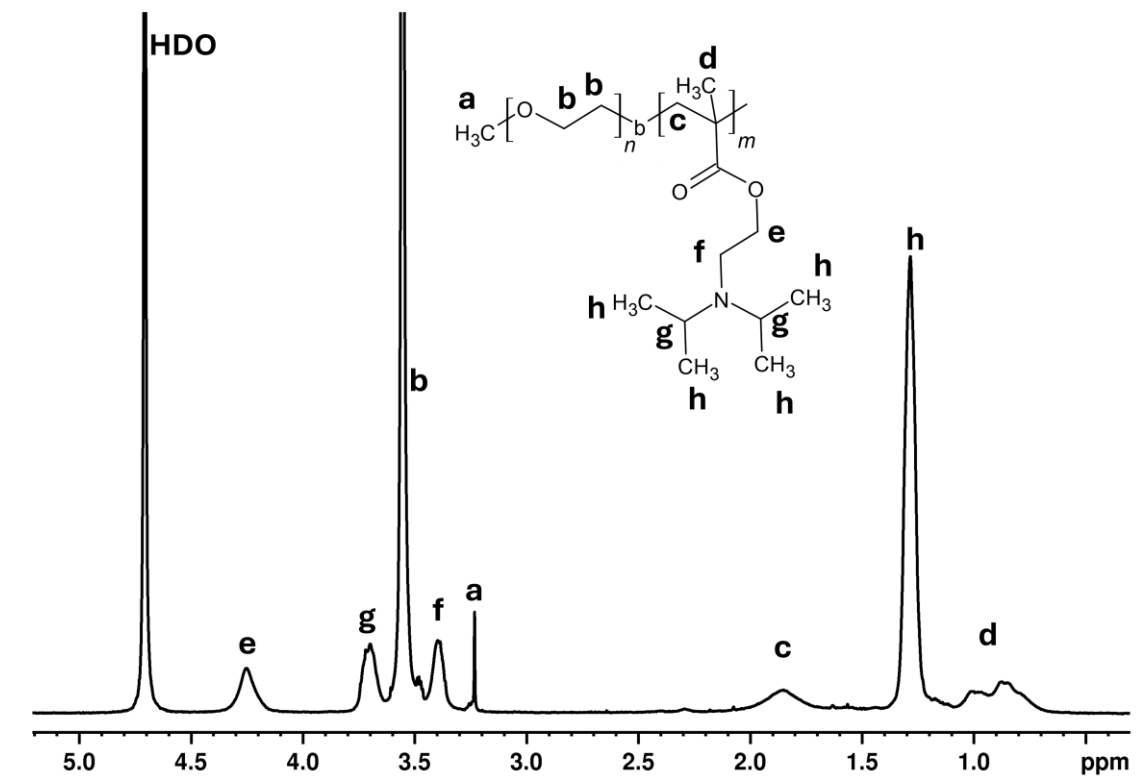


Figure 3. ¹H NMR spectrum for poly(ethylene oxide)₁₁₃-*b*-poly[2-(diisopropylamino)ethyl methacrylate]₁₈₅ (PEO₁₁₃-*b*-PDPA₁₈₅) diblock copolymer measured in D₂O/DCI (pH ~ 2).

The controlled copolymerization of the pH-sensitive block copolymers was confirmed by the low values of dispersity ($D = 1.22$ for PEO₁₁₃-*b*-PDPA₁₈₅ and $D = 1.17$ for PEO₁₁₃-*b*-PHIA₅₅) and well-defined SEC traces as reported in Figure 4.

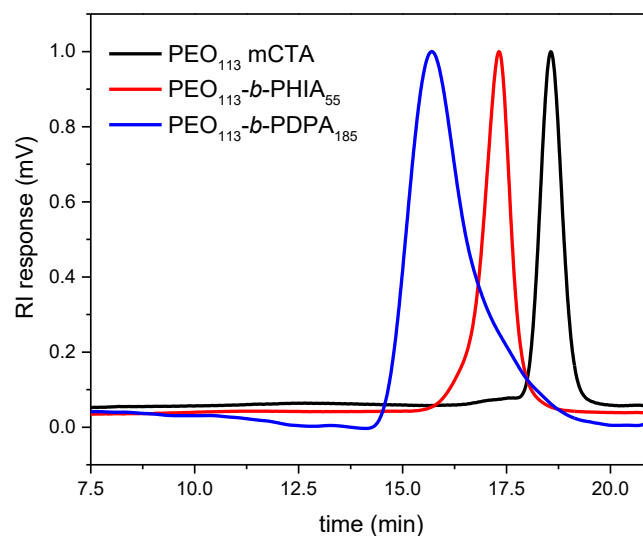


Figure 4. SEC traces for PEO₁₁₃ mCTA, and for the pH-sensitive block copolymers PEO₁₁₃-*b*-PHIA₅₅ and PEO₁₁₃-*b*-PDPA₁₈₅ obtained in chloroform-triethylamine-isopropanol 94:4:2 (v/v/v).

The manufacturing of polymer vesicles was performed by using the simple double emulsification method.³² Such protocol is highly valuable to generate polymer vesicles at the microscale. The self-assembled block copolymers were probed by using optical and fluorescence microscopy, and the microvesicles were further evaluated as microreactors by monitoring the oxidation of *o*-dianisidine in the presence of hydrogen peroxide as catalyzed by encapsulated HRP enzyme.

Firstly, the formation of the polymer vesicles using such an approach, either unloaded or loaded with Nile red was probed through optical and fluorescence microscopy, and the experimental data are provided in Figure 5. The acquired images highlight the presence of spherical vesicles at the microscale produced using the whole library of block copolymers. The accurate determination of average sizes using optical and fluorescence microscopy is challenging since all images are acquired in solution

nevertheless, the size of the polymer vesicles was estimated by measuring at least 300 vesicles roughly in the same focal plane. The results point out vesicle size ranging from 2 to 10 μm , as shown in the histograms of Figure 5C. Nile red is a well-known hydrophobic dye therefore, its presence within the spheres confirms the existence of a hydrophobic fraction in the material, corresponding to the inner layer of the membranes. This has indeed been confirmed for representative samples using confocal laser scanning microscopy. The images obtained for the pH-responsive PEO₁₁₃-*b*-PDPA₁₈₅ and PEO₁₁₃-*b*-PHIA₅₅ microvesicles reported in Figure S5 highlight the formation of the self-assemblies and the presence of Nile red embedded in the membranes.

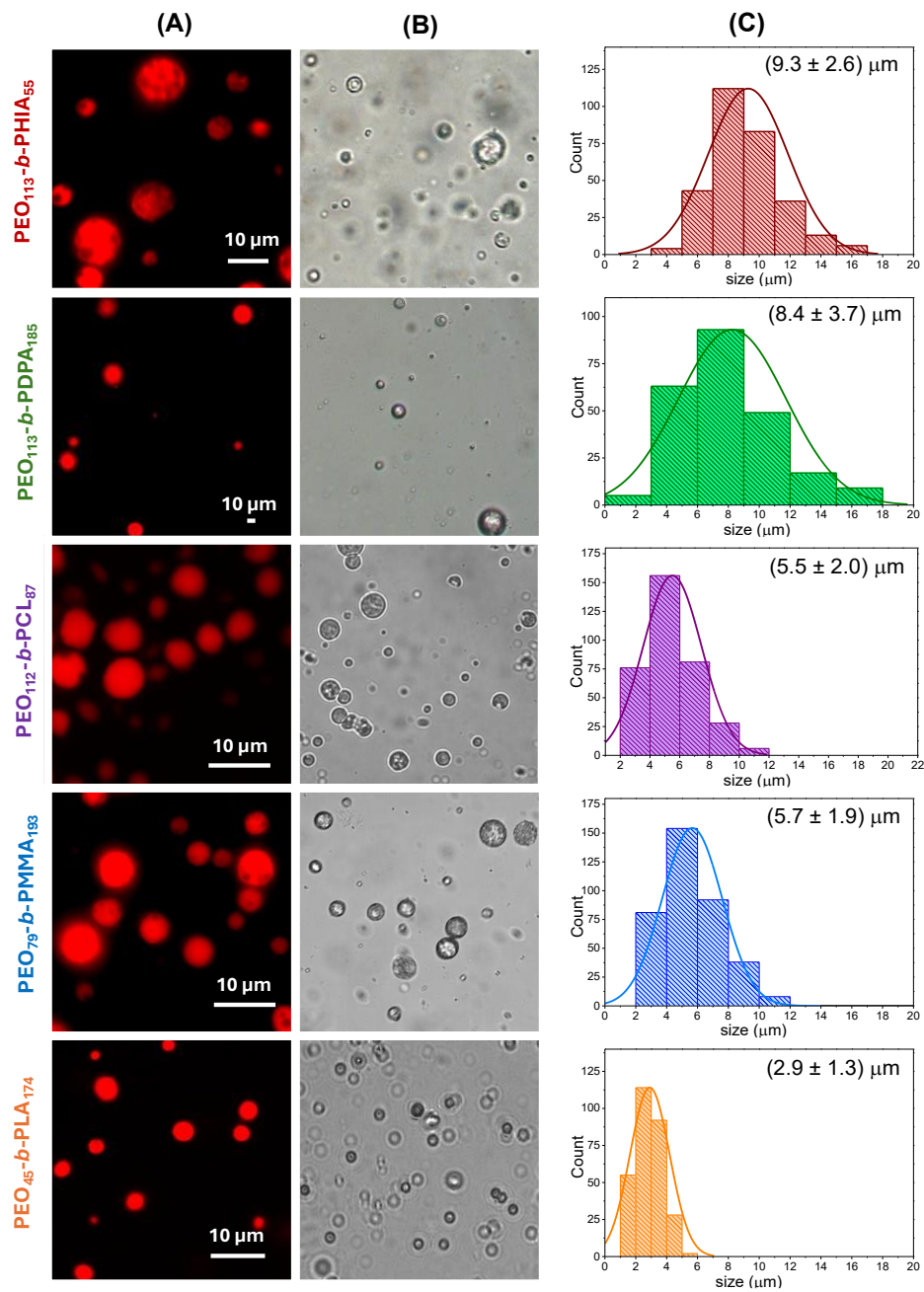


Figure 5. Images of the giant vesicles obtained by fluorescence microscopy (A) and bright-field microscopy (B) with corresponding size (diameter) histograms (mean \pm s.d.) (C).

In the step further, we have investigated the ability of the assemblies in encapsulating large proteins (including enzymes) in their aqueous lumen. This has been

conducted by using BSA labeled with a model fluorescent molecule ($M_w = 66$ kDa). The fluorescence microscopy images obtained are provided in Figure 6. This essay confirms that it is feasible to encapsulate large compounds, and thus, enzymes could be loaded towards the manufacturing of microreactors.

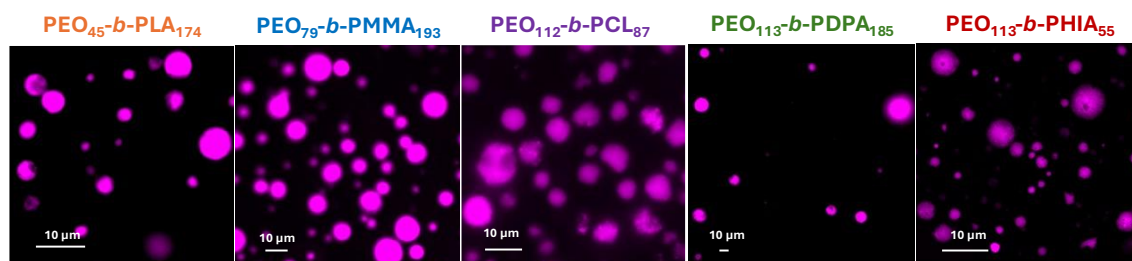


Figure 6. Images obtained with fluorescence microscopy for disparate BSA-Alexa-loaded giant polymer vesicles according to the labels.

We further investigated the feasibility of such assemblies as microreactors for biocatalysis by encapsulating the horseradish peroxidase (HRP) enzyme ($M_w = 44$ kDa) within the aqueous lumen of the giant vesicles. HRP is a peroxidase enzyme capable of catalyzing oxidation reactions of various molecules using H_2O_2 . For this assay, *o*-dianisidine (*o*-Di) was selected as the model compound and the schematic representation of the produced microreactors is provided in Figure 7A.

The substrate *o*-Di is transparent and exhibits low absorbance in solution but turns orange upon oxidation, with high absorption from 422 nm to 458 nm in the pH range from 5.0 to 8.0. The HRP catalytic activity is optimum within the pH range from 5.0 to 8.0, with maximum efficiency at acidic pH (pH 5.5).³⁵ Therefore, the assays for nonresponsive vesicles produced using $PEO_{45}\text{-}b\text{-}PLA_{174}$, $PEO_{112}\text{-}b\text{-}PCL_{87}$ and $PEO_{79}\text{-}b\text{-}PMMA_{193}$ were performed at pH 5.5. Nevertheless, as $PEO_{113}\text{-}b\text{-}PDPA_{185}$ and $PEO_{113}\text{-}b\text{-}PHIA_{55}$ disassemble in such environmental conditions, the assays had to be conducted at pH 8.0

in such cases. Yet, a comparative analysis of the HRP enzymatic activity was conducted in both pH 5.5 and 8.0 to confirm the pH-dependent differences in catalytic efficiency.

The enzyme encapsulation has been proceeded by extensive dialysis in order to remove the free (not encapsulated) fraction of HRP. Afterwards, the reactants (o-dianisidine and H₂O₂) were added at the outer compartment of the systems and the kinetics of dye oxidation was tracked by measuring the absorbance of the samples as a function of time. The set of data is given in Figure 7B and 7C.

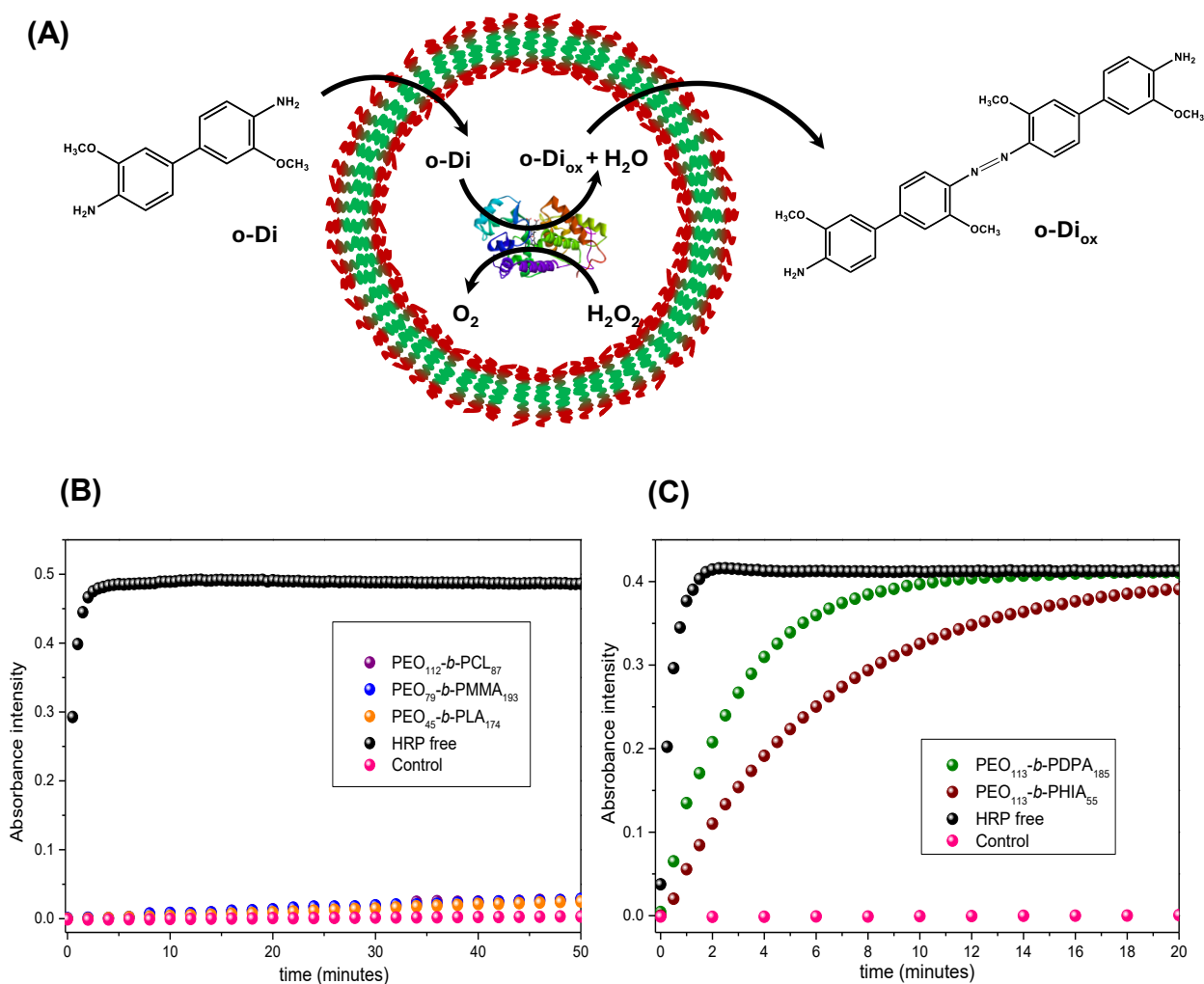


Figure 7. Schematic representation of the enzymatic cascade reaction occurring inside the polymer vesicles (microbioreactors) where HRP is horseradish peroxidase, o-Di is o-dianisidine and o-Di_{ox} is o-dianisidine in the oxidized form (A). Time-dependent

absorbance profiles obtained by UV-Vis spectroscopy for the HRP-catalyzed oxidation of o-dianisidine. The curves correspond to different HRP-loaded vesicles as indicated in the legend.

To discuss the experimental data quantitatively and considering the observed trends, the absorption profiles for the pH-responsive assemblies could indeed be fitted using an exponential growth as:

$$Abs(t) = Abs_{max}(1 - e^{(-\frac{t-t_0}{\tau})}) \quad (2)$$

where $Abs(t)$ refers to the absorbance intensity as a function of time (t), and the adjustable parameters Abs_{max} , τ , and t_0 correspond to the maximum absorbance intensity, a characteristic time, and an offset, respectively. The fitted τ values can be interpreted as characteristic times of the process; therefore, lower τ values qualitatively indicate higher reaction rates and increased vesicle permeability. The determined values of EE % for each vesicle and the τ values obtained by fitting the profiles given in Figure 7 using Equation 2 are provided in Table 1.

Table 1. Values of EE %, k , and Abs_{max} obtained for disparate HRP-loaded vesicles and free HRP.

Sample	pH	EE%	τ (min)	Abs_{max}
HRP free	5.5	-	0.51	0.487
	8	-	0.43	0.413
PEO ₄₅ - <i>b</i> -PLA ₁₇₄	5.5	78	149.35	0.028
PEO ₁₁₂ - <i>b</i> -PCL ₈₇	5.5	36	63.64	0.031
PEO ₇₉ - <i>b</i> -PMMA ₁₉₃	5.5	77	77.75	0.032
PEO ₁₁₃ - <i>b</i> -PDPA ₁₈₅	8	53	2.88	0.410
PEO ₁₁₃ - <i>b</i> -PHIA ₅₅	8	73	6.30	0.400

The first noteworthy observation is that product formation occurred particularly within the pH-responsive assemblies pointing out that these assemblies are permeable to the reaction substrate. Since the enzyme was encapsulated only within the vesicles, the diffusion of the reactants from the outer compartment of the vesicles was necessary for oxidation to occur. Secondly, the kinetics involving encapsulated enzymes were slower than those of the free enzyme, suggesting that diffusion through the membrane delayed the reaction process, as can be seen in the features reported in Figure 7, and corresponding τ values provided in Table 1. The most relevant finding is nevertheless the fact that pH-sensitive vesicles comprising a PDPA or PHIA segment are significantly more permeable than the non-responsive assemblies. Truly, polymer vesicles based on PEO₄₅-*b*-PLA₁₇₄, PEO₁₁₂-*b*-PCL₈₇ and PEO₇₉-*b*-PMMA₁₉₃ seem to have negligible permeation to the small molecule used as reactants at least in the time scale of the experiment. This behavior agrees to some extent with the recent finding by our research groups³⁶ where enhanced permeability has been evidenced for PDPA-based polymersomes, whereas PMMA-based vesicles were reported to have notably attenuated permeability to Rhodamine B used as

small model hydrophilic molecule.³⁶ The reported permeability for the pH-sensitive vesicles is attributed to the charged nature of the polymers, which facilitates the formation of hydrated layers that are supposed to enhance the diffusion of small molecules. The presence of a small fraction of charge entities within the polymeric membranes may also explain why the vesicles produced using the copolymer PEO₁₁₃-*b*-PHIA₅₅ exhibit lower permeability compared to those formed by PEO₁₁₃-*b*-PDPA₁₈₅, resulting in a slower product formation (higher τ value). In the working pH (pH = 8), the PHIA block is mainly uncharged, given its pKa of 5.5. Conversely, the PDPA block which has pKa = 6.8³⁷ still retains a considerable number of charges under these conditions, which may contribute to the formation of a more hydrated layer allowing an easier flow of substrates and products.

The nonresponsive assemblies exhibited notably low permeability even at the optimum pH (5.5). In all these cases, absorbance values remained around roughly 0.03 even after 1 hour reaction time, indicating negligible substrate conversion. The experiments were also conducted at pH 8 (not shown for brevity) however, no formation of o-Di_{ox} has been detected by UV-Vis in such condition (results shown in Fig. S6, Supporting Information). This behavior is consistent with the remarkably high τ values determined for the nonresponsive vesicles compared to the pH-responsive counterparts and free HRP. In summary, and following our early investigations in the topic,^{36,38} we highlight the intrinsically permeable nature of pH-responsive membranes based on PHIA and PDPA polymer blocks using the HRP enzyme to produce model microreactors. Giant polymer vesicles could be produced with encapsulated enzyme as evidenced by using a model protein (BSA-Alexa). Furthermore, the encapsulation of HPR and the use of a model colorful reaction demonstrate the versatility of PHIA and PDPA to produce permeable vesicles without the aid of external agents to impart permeability.

CONCLUSIONS

We herein demonstrate the manufacturing of giant polymer vesicles using the double-emulsification method with a library of block copolymers. The resulting vesicles exhibited spherical morphology with size ranging from 2 to 10 μm , as confirmed by bright-field and fluorescence microscopy. The encapsulation of model molecules such as BSA-Alexa and the enzyme horseradish peroxidase (HRP) validated the ability of these systems to host biomacromolecules in their aqueous lumen. Through oxidation assays of o-dianisidine catalyzed by encapsulated HRP in the presence of H_2O_2 , the functionality of the vesicles as microreactors was confirmed. The kinetic results revealed the permeable feature of the self-assembled pH-responsive assemblies ($\text{PEO}_{113}\text{-}b\text{-PDPA}_{185}$ and $\text{PEO}_{113}\text{-}b\text{-PHIA}_{55}$), although with reduced catalytic efficiency compared to free HRP, due to diffusion limitations across the vesicle membranes. This behavior is attributed to the partial protonation of PDPA and PHIA at the working pH, which allows for a certain degree of membrane swelling and possibly, poor polymer membrane packing. Among the probed assemblies, the PDPA-based system showed the highest permeability, likely due to its greater degree of ionization in the working pH. In contrast, non-responsive vesicles are mainly non permeable as reflected in their high τ values. Altogether, the results support the use of block copolymers containing pH-sensitive segments such as PDPA and PHIA for the rational design of vesicles with inherent permeability. These findings pave the way towards the development of functional biomimetic systems capable of operating under precise environmental conditions without the need for additional physicochemical modifications.

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