Langmuir
The ACS Journal of Surfaces and Colloids

APRIL 27, 2004
VOLUME 20, NUMBER 9

Letters

Glucose-oxidase Based Self-Destructing Polymeric Vesicles

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Received September 12, 2003. In Final Form: February 17, 2004

We have designed oxidation-responsive vesicles from synthetic amphiphilic block copolymers ("polymersomes") of ethylene glycol and propylene sulfide. Thioethers in the hydrophobic poly(propylene sulfide) block are converted into the more hydrophilic sulfoxides and sulfones upon exposure to an oxidative environment, changing the hydrophilic−lipophilic balance of the macroamphiphile and thus inducing its solubilization. Here we sought to explore generation of the oxidative environment and induction of polymersome destabilization through production of hydrogen peroxide by the glucose-oxidase (GOx)/glucose/oxygen system. We studied the encapsulation of GOx within polymersomes, its stability and activity, and glucose-triggered polymersome destabilization. Stimulus-responsive polymersomes may find applications as nanocontainers in sensing devices and as drug delivery systems.

Introduction

Encapsulation of enzymes into liposomes has been described since the first publications on the potential medical use of lipid vesicles in the early 1970s.[1−3] The immobilization, encapsulation, and entrapment of glucose oxidase (GOx), which catalyzes the conversion of β-D-glucose (glucose) in the presence of oxygen into gluconolactone and hydrogen peroxide, has attracted the interest of many research groups for potential applications in immuno-assays, glucose sensors, enzyme replacement therapy, and oral hygiene.[4−9] Incorporation of the enzyme

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10.1021/la0357054 CCC: $27.50
Published on Web 03/31/2004

in vesicles may provide protection from proteases present in biological fluids and prolong the lifetime of the enzyme by slowing denaturation processes, for example, caused by aggregation.[10−12] Furthermore, encapsulation of an enzyme such as GOx may provide controlled access to the substrate and release of the product via disruption of the barrier function of the material membrane.[6]

Winterhalter and co-workers[13] studied vesicles from synthetic copolymeric macroamphiphiles containing a channel protein inserted in the vesicle membrane and an enzyme in the vesicle interior. Both the channel protein (which regulates the enzyme substrate flow) and the enzyme retained their activity in this environment. These findings, combined with other studies on protein encapsulation into polymeric vesicles,[14] suggest that such nanocontainers are suitable for functional protein encapsulation. Moreover, the lack of charged groups in these macroamphiphiles prevents strong ionic interactions with proteins, in contrast to systems where proteins are encapsulated into cationic liposomes.[7] The added functionality (e.g., tunable thickness of the membrane and thus tunable permeability) of vesicles formed from polymeric components, also termed polymersomes,[15,16] has attracted our interest toward the development of macroamphiphiles able to assemble in water into vesicular structures and possessing the additional feature of inducible degradability.

Recently, some of us have described a versatile and mild synthetic method for the preparation of ABA triblock copolymeric macroamphiphiles in which the hydrophobic B block consists of poly(propylene sulfide) (PPS) and the two hydrophilic A blocks consist of poly(ethylene glycol) (PEG).[17] These copolymers form lyotropic lamellar mesophases in water and, upon dilution, stable vesicles.[18,19] In our design of this class of macroamphiphiles, the sensitivity of the PPS hydrophobic block to oxidation, resulting in the conversion of the thioethers into sulfoxides and sulfones, is a key feature. Exposure of the initially stable polymersomes to an oxidative environment results in their destabilization and thus release of their contents.[20]

In this communication, we report on the encapsulation of GOx into PEG−PPS−PEG polymersomes, on the stability and activity of the enzyme in this system, and on the polymersomes' membrane properties in terms of substrate permeation and protein interaction. In PEG−PPS−PEG polymersomes, incorporation of an oxidant-generating enzyme and use of a membrane permeable substrate has the potential to induce triggered polymersome destruction after extravesicular addition to the substrate. Indeed, glucose permeation through the polymersome membrane resulted in intravesicular H_2O_2 generation, inducing polymersome destabilization, thus providing a model of release triggered by an environmental stimulus.

Experimental Section

Block Copolymer Synthesis. The PEG−PPS−PEG block copolymer under study was prepared as previously described.[17] The symmetric $EG_{16}PS_{50}EG_{16}$ triblock copolymer used herein (theoretical structure from the feed) had a polydispersity index (PI) of 1.3 and a M_n of 5300 Da (EG weight fraction, $f_{EG} = 0.28$).

Vesicle Preparation. PEG−PPS−PEG block copolymer (10 mg) was placed in a 10 mL round-bottom flask, and 1 mL of PBS (0.01 M, pH 6) was added. This buffer was chosen because it offers a good compromise between enzyme stability and enzyme activity.[21] The polymer film (at room temperature, the polymer is a viscous liquid) was left to hydrate by rotating the flask in a warm bath (∼35 °C). After 15 min, the suspension was vortexed and freeze−thawed several times to assist in homogenization. After this preparation step, the main components of the 1 wt % copolymer suspension are large multilamellar vesicles. To reduce the lamellarity and the size distribution of the vesicles formed, the suspension was extruded 20 times through a 200 nm pore-size polycarbonate filter using a miniextruder (Avanti Lipids, Alabaster, AL).

Vesicle Oxidation Experiments. A vesicle suspension in PBS was diluted to obtain an optical density (OD) of 1.5 (AU) at 600 nm and placed in a dialysis membrane (2 cm diameter, 14 kD molecular weight cutoff (MWCO)). The membrane was placed in a 100 mL cylinder containing PBS at pH 6.0 (0.01 M), GOx (∼160 kDa)[10,22] 0.3 mg/mL, and previously mutarotated glucose (0.1 M). The ratio GOx/glucose was chosen such that in 1 h the solution would contain ca. 0.3% H_2O_2. Air was bubbled through the solution outside the membrane; the membrane prevents direct contact between the vesicles and the enzyme. Glucose solution (1 M) was added in 4 aliquots of 10 mL during the first 72 h of the experiment. A funnel was inserted in the upper part of the membrane in order to have access to the vesicle suspension via tubing connected to a flow cell in the UV−vis spectrometer (Perkin-Elmer Lambda 20). The optical density of the vesicle suspension was monitored over time. For the blank, the samples were prepared excluding the presence of the enzyme.

Encapsulation of GOx into Polymersomes. The preparation of GOx-encapsulated vesicles follows the protocol described above where instead of pure PBS a 0.3 mg/mL solution of GOx from *Aspergillus niger* (Sigma Aldrich, U.S.) in the same buffer was used. The freeze−thawing of the suspension has been excluded to avoid enzyme denaturation.

Purification of GOx-Encapsulated Vesicles. To remove the free enzyme from the GOx-containing vesicles, we purified by size exclusion chromatography (SEC) using an integrated Äkta FPLC (Amersham) equipped with a column Superdex 200 HR 10/30 (Amersham). Fractions of 0.5 mL were collected at a flow rate of 0.4 mL/min and an average pressure of 0.65 bar and monitored with a UV spectrometer at $\lambda = 280$ nm.

Enzymatic Activity Measurements. GOx activity was assayed by a standard hydrogen peroxide determination in PBS (0.01 M, pH 6.0) using horseradish peroxidase (HRP, 4.54×10^{-10} M) (Sigma), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 4×10^{-7} M), and glucose (3×10^{-4} M, physiological glucose concentration is in the range of $5-10 \times 10^{-3}$ M) while bubbling with air. The increase in absorbance at $\lambda = 420$ nm, given by ABTS radical formation, was monitored for 60 s. When testing HRP activity in the presence of polymersomes, we found that the enzyme activity is not affected in the polymersome concentration range of interest, suggesting

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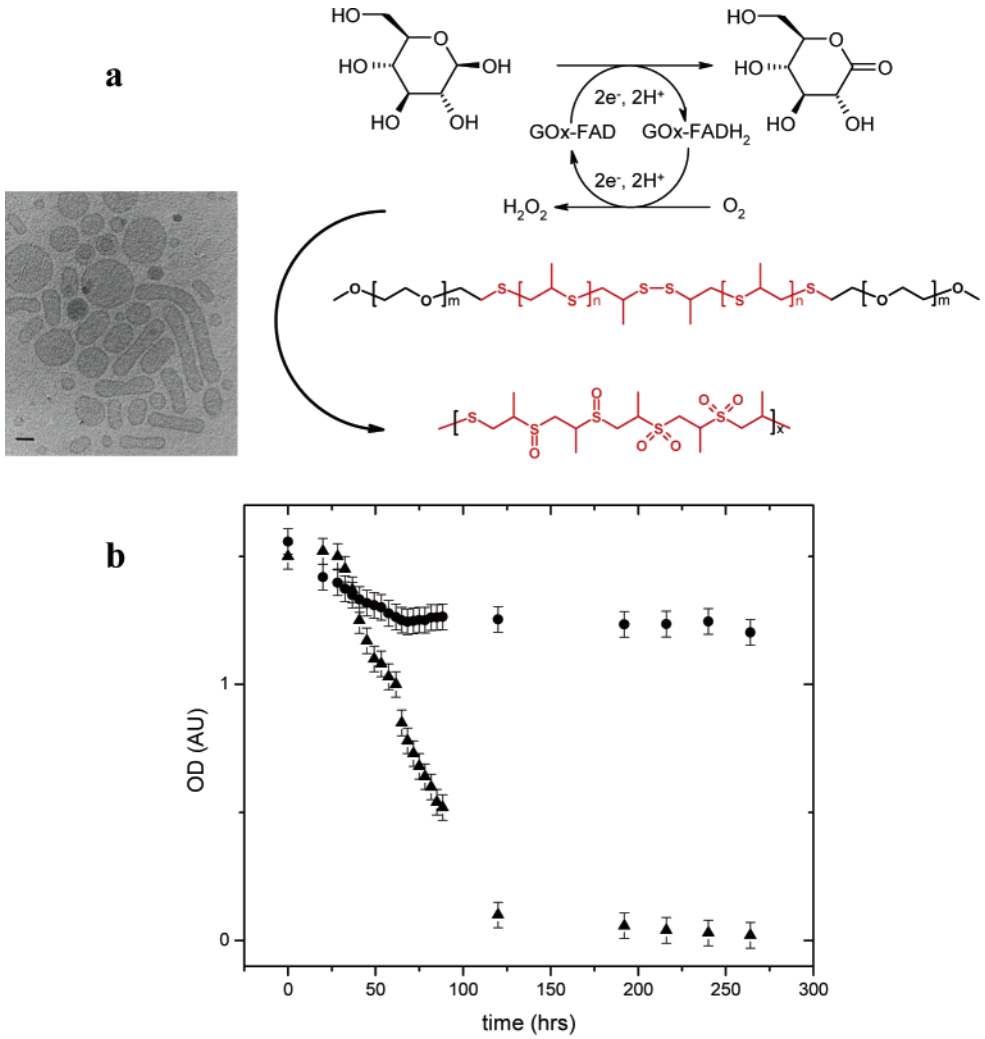

Figure 1. (a) The GOx-catalyzed conversion of β-D-glucose into gluconolactone with production of H_2O_2 in the presence of oxygen. Below the symmetric PEG−PPS−PEG block copolymer under study and a possible repeating unit of PPS after oxidation by H_2O_2, $m = 16$, $n = 25$. In the inset, a cryo-TEM micrograph of the polymersomes formed in buffer after extrusion (the bar represents 100 nm). (b) Turbidity measurements of PEG−PPS−PEG polymersomes showing degradation in the presence of extravesicular GOx (▲) and the same experiment (●) without GOx. The suspension was kept separated from the enzyme using a dialysis membrane (14 kDa MWCO). The OD of the solution at $\lambda = 600$ nm was monitored over time. The initial decrease in OD for the control experiment might be associated with partial sensitivity of the thioethers in the hydrophobic block to dissolved molecular oxygen.

a faster reaction of hydrogen peroxide with ABTS and HRP than with the PPS block of the polymer.

Destabilization of the GOx-Encapsulating Polymersomes. A suspension of purified GOx-encapsulating polymersomes was placed in a vial containing aqueous 0.1 M glucose solution. Prior to OD measurements at $\lambda = 600$ nm, air was bubbled through the solution. As a blank, the same experiment was conducted with the polymersome fraction of a purified mixture of preformed polymersomes and GOx.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Copper grids (300 mesh) coated with a perforated carbon film according to a modified procedure described by Fukami and Adachi[23] were used for the preparation of the frozen hydrated polymersome suspension specimen by the bare-grid technique.[24] The grids were not treated by glow discharge to keep them hydrophobic. Thin aqueous films were prepared at room temperature (RT) and controlled relative humidity (97−99%)[25] within a custom-built environmental chamber as follows. A total of 10 μL of the vesicle suspension was applied to the grid. The excess liquid was blotted with filter paper for 2−3 s, and the residual thin aqueous films remaining in the pores of the carbon film were rapidly vitrified by plunging the grid into liquid ethane. The grids were mounted in a Gatan cryoholder and examined in a Zeiss EM 912 OMEGA microscope, equipped with an energy filter, at 120 kV and at a specimen temperature of ca. 105 K. Electron micrographs were digitally recorded using a cooled 1024 × 1024 CCD camera (Proscan, Germany).

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Results and Discussion

The destabilization of polymersomes of $EG_{16}PS_{50}EG_{16}$ (Figure 1a) induced by hydrogen peroxide produced with GOx-catalyzed conversion of glucose into gluconolactone was first studied keeping the polymersome suspension separated from the enzyme with a dialysis membrane. In this way, interactions between the enzyme and the polymersomes, which could potentially disrupt the enzyme's function or directly destabilize the polymersome, are excluded.

The vesicle suspension (Figure 1a, inset) was placed within a dialysis membrane (14 kDa MWCO), which was inserted in PBS with GOx and glucose, through which air was bubbled. The production of H_2O_2 by the system GOx/glucose/oxygen turned the initially turbid vesicle suspension into a clear solution, as shown in Figure 1b. Oxidation of the PPS thioethers (Figure 1a) into sulfoxides and

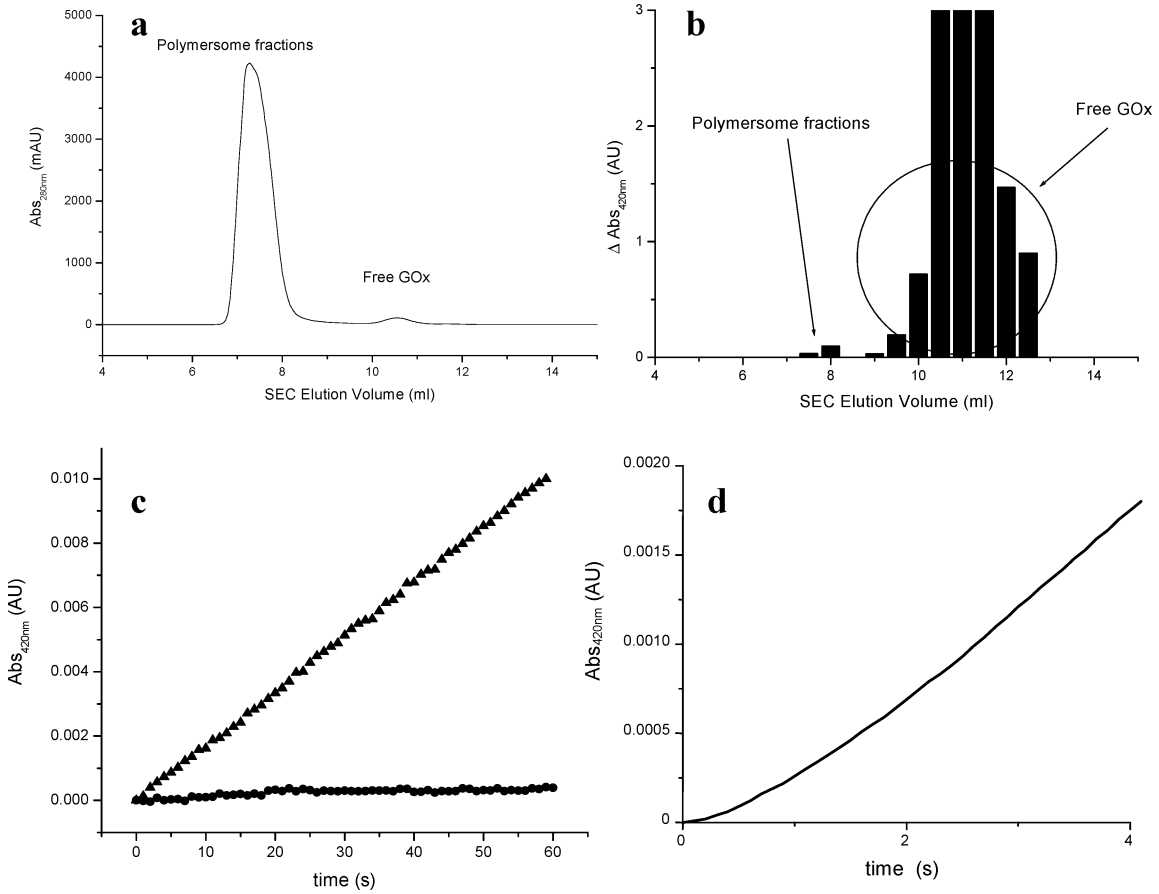

Figure 2. (a) The SEC elution profile of GOx-encapsulating polymersomes and (b) the corresponding measured activity of the collected fractions. The GOx-encapsulating polymersomes have much lower activity compared to the free GOx fractions, since the amount of encapsulated GOx is limited by the volume fraction enclosed by polymersomes in this dilute sample preparation. (c) Activity curves of the polymersome fractions of SEC-purified suspensions of polymersome-encapsulated GOx (▲) and of polymersomes with extravesicular GOx (●). The absence of detectable activity in the second case suggests the lack of GOx adsorption on the surface of the polymersomes. (d) The activity curve for the encapsulated GOx in the first 4 s. A slight delay in response can be noticed, which may be attributed to glucose diffusion into the polymersome membrane and H_2O_2 diffusion out of the membrane.

sulfones induces conversion of the initially amphiphilic PEG−PPS−PEG copolymer into a hydrophilic polymer, resulting in the destabilization of vesicles. We have investigated this process under even more drastic oxidative conditions (10 vol % H_2O_2)[20] and observed that polymersome destabilization occurs via morphological changes without scission of the block copolymer. The kinetics of polymersome destabilization is influenced by the concentration of hydrogen peroxide at equilibrium and its permeation through the dialysis membrane. Enzyme and glucose concentrations (the latter was added in aliquots during the experiment) were adjusted to yield a H_2O_2 concentration near 0.3 vol %.

Subsequent experimentation was conducted with GOx encapsulated within the PEG−PPS−PEG polymersomes. Encapsulation of GOx within these polymersomes was achieved using standard film hydration methods using a 0.3 mg/mL GOx solution, avoiding freeze−thawing cycles to preserve enzyme integrity.[26] The effect of vortexing (used in polymersome formation) was tested on GOx solutions and did not show any effect on enzymatic activity. The critical step of purification of free GOx from the GOx-encapsulated fraction was successfully achieved by SEC, as can be seen in Figure 2. The polymersome-encapsulated and free enzyme fractions were tested for GOx activity using an assay with ′ABTS, HRP, glucose, and O_2. These two fractions could be efficiently separated by size exclusion chromatography, as shown in Figure 2a,b. Polymersomes without encapsulated enzyme showed no activity.

To exclude the possibility that activity observed for the polymersome-associated fractions derives from enzyme adsorbed onto the vesicle outer surface, we incubated preformed polymersomes with GOx and measured the activity of the polymersome fractions after purification by SEC. As can be seen from Figure 2c, no enzymatic activity could be detected for these samples, thus allowing all polymersome-associated activity to be attributed to encapsulated enzyme.

The activity measurements of GOx-encapsulating polymersomes, shown in Figure 2c, demonstrate clearly that glucose diffuses into polymersomes. Transport of glucose through the polymersome membrane is dependent on the solubility and diffusion coefficient of glucose in the PPS block, which ultimately affects its permeation coefficient across the membrane. The PPS block is characterized by a low glass transition temperature (∼230 K) and is therefore highly mobile at room or body temperature; increased permeability of glucose and other nonionic permeants has also been observed in liposomes with very fluid lipid membranes, that is, which are above their liquid crystal transition temperature (T_c), while in the presence of a T_c-increasing rigid molecule like cholesterol, perme-

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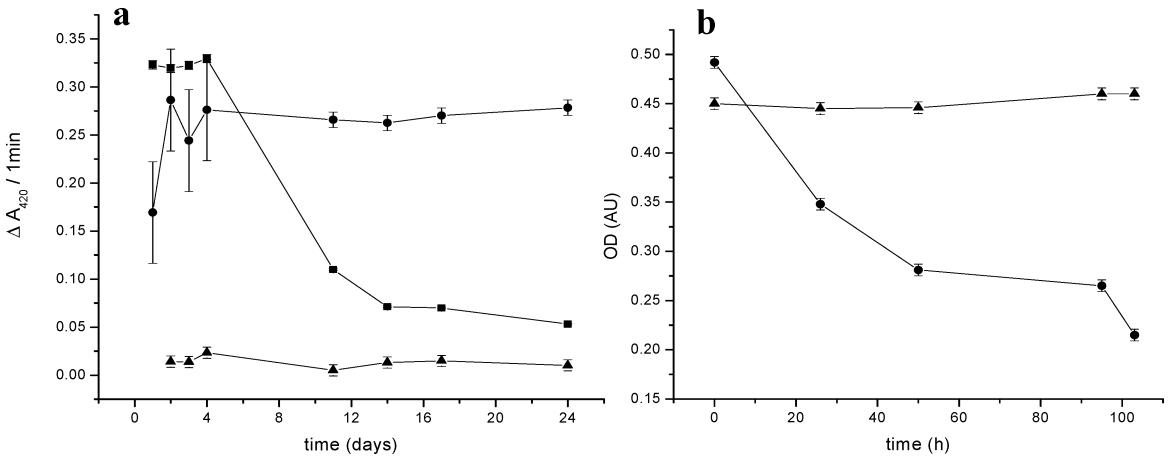

Figure 3. (a) The observed activity for GOx (at room temperature) was dramatically extended by encapsulation in polymersomes (▲) and in their presence (●) compared to free GOx (■). While the free enzyme starts to lose activity after 5−6 days, the GOx in the presence of or encapsulated by polymersomes retains its activity for at least 24 days. (b) Turbidity measurements vs time at $\lambda = 600$ nm on GOx-encapsulating vesicles in the presence of glucose (0.1 M) and oxygen (●) and of empty vesicles under the same conditions (▲).

ability has been observed to decrease.[27−29] At the extravesicular glucose concentration used in these experiments (0.1 M), a minimal delay of ca. 0.5 s was observed between glucose addition and ABTS oxidation (determined from the absorbance increase at 420 nm, Figure 2d). These findings also highlight the fast diffusion of H_2O_2 into the PPS domain, suggesting that the thioethers in the hydrophobic regions of the membrane can be easily accessed by the oxidant.

A benefit to the stability of GOx was observed to result from polymersome encapsulation. Aliquots of the GOx-encapsulating polymersome suspension were assayed over time by measuring the increase in absorbance at $\lambda = 420$ nm for the initial 60 s of each measurement. The same was done for a solution containing only GOx and for a solution of GOx mixed with preformed polymersomes (Figure 3a). GOx activity was dramatically stabilized, both within polymersomes as well as in their presence. This result suggests that the presence of PEG−PPS−PEG colloids protects the enzyme from hydrophobically induced denaturation, whether by aggregation or contact with hydrophobic surfaces, and may induce a higher structural rigidity of the enzyme by steric repulsions in a fashion similar to that by which carbohydrates reduce the fluctuation amplitude of the enzyme's flexible side chains.[4,30]

A key question to us is whether GOx is capable of destabilizing its polymersome container in response to extravesicular addition of glucose. Indeed, this was observed, as can be seen in the turbidimetry measurements shown in Figure 3b.

From these measurements alone, it is not possible to gather information about the morphological changes occurring in this dispersion; however, it is clear that the overall result is an increased solubility of the polymer. By comparison with our studies of effects of direct exposure to H_2O_2, it is likely that the polymersomes transition to wormlike micelles, spherical micelles, and unimolecular micelles as the initially hydrophobic PPS domain is oxidized into the more hydrophilic sulfoxide- and sulfone-containing polymer.

In this work, we sought a vesicle system that was highly sensitive to environmental stimuli, modulating destabilization of PEG−PPS−PEG vesicles by exposure to oxidative conditions. Intravesicular GOx served as the transducer from extravesicular glucose stimulation, the glucose diffusing across the hydrophobic polymersome membrane to act as a substrate for GOx, yielding H_2O_2 as a reaction product. Although polymeric systems responsive to pH changes induced by the couple glucose/GOx have been reported,[31] here we introduce a novel mechanism based on oxidation-sensitive copolymers. These materials and this approach may have utility in drug delivery and detection and sensing of biological analytes.

Acknowledgment. M.J.B., N.A.J.M.S., and R.J.M.N. thank the National Research School Combination Catalysis (NRSC-Catalysis) for support of this work. A.N., N.T., and J.A.H. acknowledge financial support from the Research Commission of the ETHZ. A.N. and M.J.B. are grateful to Cornelia Weber for her assistance in the SEC polymersome purification. Martin Müller of the EM Lab at ETH Zurich is gratefully acknowledged for cryo-TEM measurements.

LA0357054

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