

Phospholipid-Based Catalytic Nanocapsules**

By Glenn E. Lawson, Yongwoo Lee, Frank M. Raushel, and Alok Singh*

The encapsulation and catalytic efficiency of organophosphate hydrolyzing enzymes in polymer-stabilized nanocapsules is reported. Polymerized vesicles—derived from a headgroup-polymerizable phospholipid, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-(2-hydroxymethyl)-3,5-divinylbenzamide (DPPE-DVBA)—containing enzymes were used as catalytic nanocapsules. Three enzymes, organophosphorus hydrolase (OPH), phosphotriesterase (PTE), and organophosphorus acid anhydrolase (OPAA), were encapsulated in vesicles by incubating them with freeze-dried vesicles at 55 °C, followed by intermittent vortex-mixing. Enzyme-containing vesicles, collected after gel-filtration, were stabilized by photopolymerization at 254 nm to yield crosslinked catalytic nanocapsules. The nanocapsules containing OPH and PTE showed specific activities of 0.36 and 1.74 $\mu\text{mol mg}^{-1} \text{min}^{-1}$, respectively, against methyl parathion (MPT), and OPAA-containing nanocapsules showed a specific activity of 57.1 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ against diisopropylfluorophosphate. Freeze-dried, OPH- and PTE-containing nanocapsules showed retentions of 83 % and 85 % specific activity, respectively, upon redispersion in buffer solution. Three-week, room-temperature storage of OPH-containing nanocapsules showed a retention of 18 % enzyme activity. Hydrolysis of MPT in crosslinked DPPE-DVBA/OPH vesicles showed that hydrophobic MPT permeated through the bilayer membrane of the freeze-dried nanocapsules, releasing the hydrolysis product *para*-nitrophenol, which permeated back to the exogenous dispersion medium leaving the enzymes free to react with freshly permeated MPT in the interior of the nanocapsules.

1. Introduction

Phospholipid-based polymerized vesicles belong to a unique class of multifunctional materials. Their crosslinked membranes provide a stable hydrophilic interface that is permeable, with a hydrophobic interior and a refillable central cavity. Hence, they offer ample opportunities for developing new applications. Our recent results on vesicle stabilization under milder conditions^[1] led us to explore the unconventional utility of the vesicles as reusable catalytic nanocapsules. In nature, lipid–protein interactions are ubiquitous. Therefore, we chose to explore the encapsulation of enzymes within the interior of vesicles to investigate if their catalytic activity was retained. It is hoped that by encapsulating the enzymes in this way, the active enzymes could be readily transported to desired sites. Enzymes have been widely used for passivating chemical agents because of their high catalytic turnover rates and specificity.^[2,3] Phospholipid vesicles have previously been used for encapsulating enzymes within their interior aqueous compartment,^[4–6] or interlamellar spaces.^[7] Enzyme immobilization, either covalent^[8] or electrostatic,^[9] on the surfaces of vesicles, has also

been studied. The entrapment of enzymes in reverse micelles has also been reported in a variety of applications.^[10] Non-phospholipid-based systems have included alginate-based capsules,^[11] sol–gels,^[12] polyurethane particles,^[13,14] and polyelectrolyte multilayers.^[15] However, each of these systems has drawbacks, which range from the loss of enzyme activity—stimulated by the reaction conditions—to a lack of accessibility of the substrates to the enzyme. The approach of using the core of small, unilamellar vesicles is appealing because phospholipid-based vesicles mimic natural biological cells. They therefore afford a biodegradable protective carrier system which acts as a both a three-dimensional support to and natural biological habitat for the enzymes. A disadvantage, however, is that the vesicles disintegrate after a certain period of time, thereby retarding the enzyme's activity. Another problem is that conventional, non-polymerizable vesicles are also unstable to freeze-drying/redispersion cycles and therefore have a limited applicability. These shortcomings have been overcome by crosslinking individual phospholipid molecules without affecting their membrane functions.^[1]

Our goal was to explore the catalytic activity and stability of enzymes encapsulated within polymerized vesicles. We selected three enzymes: organophosphorus hydrolase (OPH), phosphotriesterase (PTE), and organophosphorus acid anhydrolase (OPAA) for encapsulation in vesicles derived from 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-(2-hydroxymethyl)-3,5-divinylbenzamide (DPPE-DVBA).^[1] Figure 1 schematically illustrates the reaction between the organophosphate compound methyl parathion (MPT) and an encapsulated enzyme. The progress of the enzyme-catalyzed hydrolysis reaction was followed by monitoring the production of *para*-nitrophenol (PNP). Additionally, the formation and stability of the crosslinked vesicles against organic solvents has been evaluated.^[16]

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[**] Financial support for this work came from the Office of Naval Research through an NRL base program. G.L. is an NRC research associate, and Y.L. is an ASEE fellow. We thank T. C. Cheng for the OPAA testing.

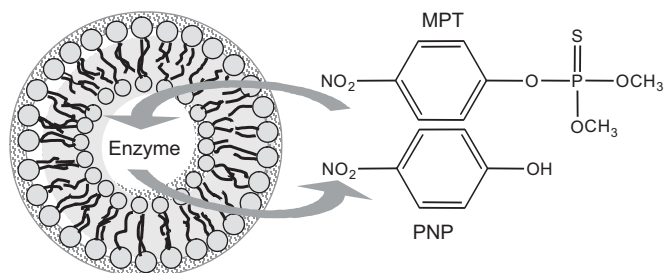


Figure 1. Schematic representation of MPT hydrolysis catalyzed by enzyme-containing poly(DPPE-DVBA) vesicles. The hydrolysis product, PNP, diffuses out from the vesicles to the surrounding medium.

2. Results and Discussion

We have recently reported the formation of vesicles from a novel phospholipid with 1,2-divinylbenzoyl functionality in the headgroup region.^[1] Vesicles formed from this class of phospholipids underwent complete polymerization, induced by a combination of photo-irradiation and radical initiator. Material encapsulation, permeability, stability, and enzyme-activity studies on the vesicles were performed before and after polymerization, which was followed by freeze-drying and redispersion steps. Three enzymes, OPH, PTE, and OPAA, were used in this study in order to achieve a broad range of activity against organophosphate compounds. The structures of OPH and PTE are similar. We chose OPH-containing, crosslinked, freeze-dried vesicles for MPT hydrolysis, with possible application in mind. The OPH used in this study was 5% OPH and $\approx 95\%$ trehalose (along with some small amounts of associated protein), as trehalose is known to stabilize freeze-dried enzymes in the solid state. Thus, we used 5% OPH, which is both economically and technologically attractive; the overall hydrolysis capacity of the enzyme is unaffected.

2.1. Visualization

Figure 2 provides visual proof of the presence of vesicle structures in sonicated lipid suspensions in water. Transmission electron microscopy (TEM) images clearly show the presence

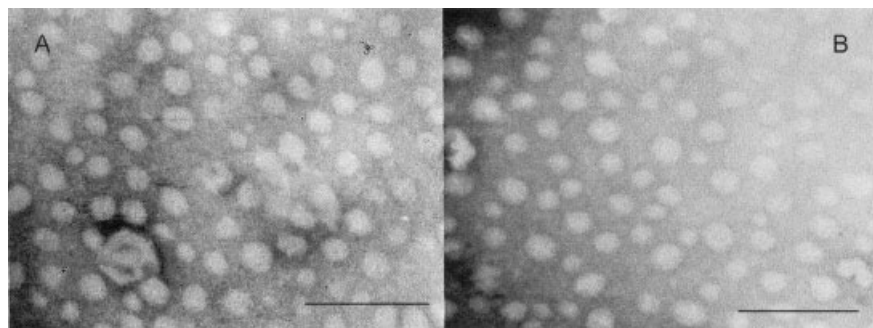


Figure 2. Transmission electron microscope images of OPH-containing vesicles: A) DPPE-DVBA vesicles before crosslinking; B) DPPE-DVBA vesicles after crosslinking. The scale bars represent 250 nm.

of almost uniformly sized vesicles in the dispersions. Both non-polymerized (Fig. 2A) and polymerized (Fig. 2B) vesicles from the DPPE-DVBA lipid had an average diameter of 60 nm. Vesicle sizes differed from preparation to preparation, but the average size remained the same. Characterization of the vesicle size and shape before and after polymerization shows that the crosslinking method is truly non-intrusive for making stabilized structures.

2.2. Encapsulation of Enzymes in Vesicles

The vesicles for enzyme encapsulation were prepared by sonicating the hydrated lipid until a constant turbidity was observed (UV-vis spectroscopy showed an unchanging absorbance of 0.02 (a.u.) at 400 nm). The small, unilamellar vesicles thus produced were freeze-dried and redispersed after adding OPH dissolved in BTP-Co (Bis Tris Propane (10 mM)–CoCl₂ (100 μ M)) buffer at pH 8.6. Hydration at 55 °C and vortex-mixing produced small vesicles. It has been reported that vesicles produced from freeze–thaw cycles form uniform dispersions with high encapsulation efficiencies.^[8] Partitioning a lipid in a chloroform/methanol/water medium, and removing the organic solvent using a rotary evaporator, has resulted in the formation of giant, unilamellar vesicles.^[9] Giant vesicles are ideal for encapsulating a higher concentration of large molecules. We did not use either technique, because of the potential for enzyme deactivation by repeated freeze–thaw cycles or organic solvents. The current method of redispersion of freeze-dried vesicles was chosen because of the ease of vesicle preparation and to avoid the potential deactivation of the enzymes. The enzyme OPH was selected because of its potential application in pesticide detoxification. A profile of vesicle separation from the exogenous protein was made by monitoring each fraction collected during gel filtration by UV-vis spectrophotometry, as shown in Figure 3. A graph was plotted of the absorption of OPH-containing vesicles at an absorption maximum of 263 nm (PTE at 279 nm and OPAA at 290 nm) versus fraction number. The absorption maximum of the vesicles was monitored at 400 nm versus fraction number. Enzymes encapsulated in vesicles were measured as a simultaneous absorption of enzyme and vesicles. The presence of protein in the fractions containing vesicles is indicative of the existence of enzyme-containing vesicles. The encapsulation efficiency was optimized for crosslinked vesicles containing OPH. The highest encapsulation percentages were found to be 24% ([OPH] = 600 μ g mL⁻¹), and 30% ([OPH] = 200 μ g mL⁻¹). The encapsulated efficiency for crosslinked PTE-containing vesicles was found to be 48% ([PTE] = 200 μ g mL⁻¹). These results indicate that encapsulating solutions with higher enzyme concentration do not increase the encapsulation efficiency.

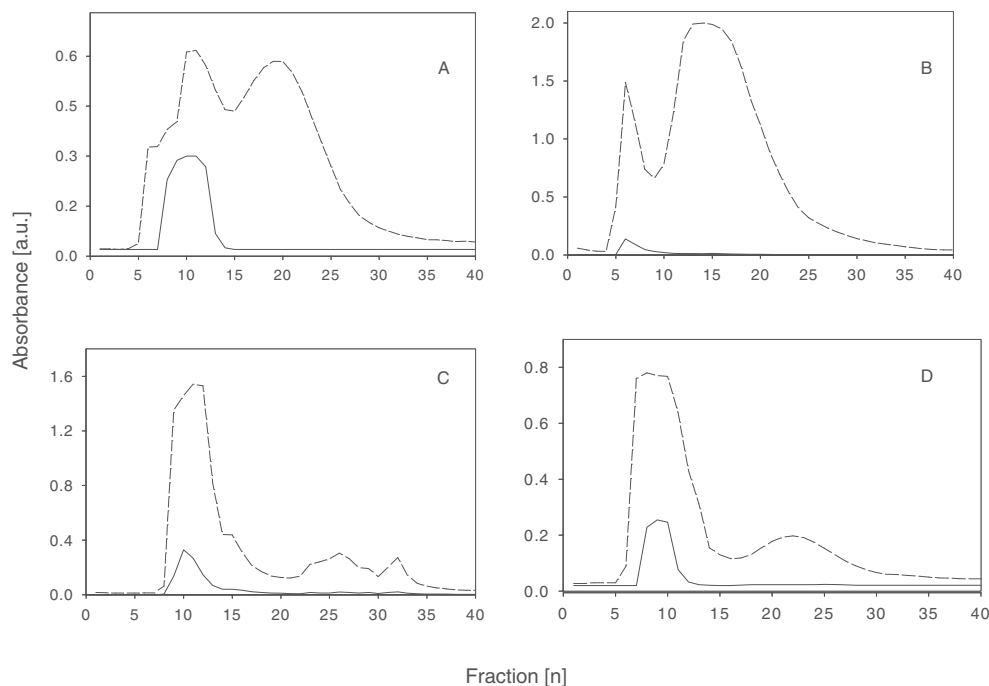


Figure 3. Gel-filtration separation profiles monitored using UV-vis spectra of enzymes encapsulated in DPPE-DVBA vesicles. A) [OPH] = 100 $\mu\text{g mL}^{-1}$; B) [OPH] = 300 $\mu\text{g mL}^{-1}$; C) [PTE] = 100 $\mu\text{g mL}^{-1}$; D) [OPAA] = 100 $\mu\text{g mL}^{-1}$. Fractions ≥ 20 represent free enzyme. Dashed line: absorption due to enzyme. Solid line: absorption due to vesicles.

2.3. Enzyme Activity

Both crosslinked and non-polymerized enzyme-containing vesicles were brought in contact with pesticide (MPT). The presence of MPT and the progress of the MPT hydrolysis was observed at room temperature using spectrophotometry. The generation of hydrolysis product, PNP, was monitored at 405 nm for 120 s and the rate of enzyme catalysis was calculated. In Table 1, the data for gel-filtered, enzyme-containing vesicles before polymerization show specific activities [$\mu\text{mol mg}^{-1} \text{min}^{-1}$] of 0.55 ([OPH] = 300 $\mu\text{g mL}^{-1}$) 0.50

([100 μg] = mL^{-1} OPH), and 2.93 ([PTE] = 100 $\mu\text{g mL}^{-1}$). The specific activities calculated after polymerization (via UV irradiation) were 0.40 (300 $\mu\text{g mL}^{-1}$ OPH), 0.36 (100 $\mu\text{g mL}^{-1}$ OPH), and 1.74 (100 $\mu\text{g mL}^{-1}$ PTE) $\mu\text{mol mg}^{-1} \text{min}^{-1}$. The retention of enzyme activity after crosslinking is shown in Table 1 for each of the systems. To determine the extent of enzyme deactivation by UV irradiation, the free enzyme (OPH or PTE) in solution was exposed to UV light for 5 min: a 62 % reduction in activity for the OPH enzyme and 80 % reduction for PTE were found. Experiments are in progress to minimize the deactivation of the enzymes during polymerization by changing the vesicles' size and lamellarity. To determine the effect of freeze-drying on the activity of the enzyme, crosslinked, freeze-dried vesicles were redispersed in BTP-Co buffer (100 μM ; pH 8.6) for OPH-containing vesicles; an 83 % retention in specific activity was found. Redispersed, freeze-dried, crosslinked vesicles containing PTE showed an 85 % retention of specific activity. The crosslinked enzyme-containing vesicles were tested for stability and activity over long periods of time. After storage for three weeks at room temperature, freeze-dried, crosslinked vesicles containing OPH were redispersed in 100 μM BTP-Co buffer (pH 8.6) and examined for their hydrolytic activity against MPT. The specific activity was found to be 0.064 $\mu\text{mol mg}^{-1} \text{min}^{-1}$, which is 18 % of the specific activity found in the freshly prepared crosslinked vesicles. The crosslinked vesicles containing OPAA showed a value of 57.1 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ against diisopropylfluorophosphate (DFP) after freeze-drying and storing at room temperature for three weeks.

Table 1. Catalytic activity of OPH and PTE at room temperature before and after encapsulation in DPPE-DVBA vesicles.

	Concentration of enzyme in encapsulating solution [$\mu\text{g mL}^{-1}$]		
	OPH, 300	OPH, 100	PTE, 100
Specific activity in non-crosslinked vesicles [$\mu\text{mol mg}^{-1} \text{min}^{-1}$]	0.55	0.50	2.93
Specific activity of enzyme in crosslinked vesicles [$\mu\text{mol mg}^{-1} \text{min}^{-1}$] [a]	0.40	0.36	1.74
Retention of specific activity of crosslinked vesicles [%]	73	71	60
Retention of specific activity of crosslinked vesicles after freeze-drying [%]	–	83	85

[a] Retention of specific activity of free enzyme after 5 min exposure to UV light: OPH, 38%; PTE, 20%.

2.4. Hydrolysis of MPT in Crosslinked DPPE-DVBA/OPH Vesicles

The catalytic activity of OPH encapsulated in freeze-dried, crosslinked vesicle powder was compared to the catalytic activity of OPH encapsulated in crosslinked vesicles in solution. The activity of freeze-dried, crosslinked DPPE-DVBA vesicles with encapsulated OPH as a powder was determined by monitoring the hydrolysis of MPT to PNP at room temperature. In Figure 4A, control experiments show that without free enzyme present in solution or encapsulated in vesicles, MPT is not hydrolyzed to PNP. The activity of the freeze-dried vesicles with encapsulated OPH was studied using MPT concentrations of 6.25, 12.5, 25, 100, and 187.5 μM . As shown in Figure 4A, for MPT concentrations of 6.25, 12.5, and 25 μM the overall hydrolysis steadily increases for the first four hours, after which the production of PNP levels off. In the first three hours, the initial rate was found after calculation of the slope by linear regression. All three data curves had a squared correlation coefficient of 0.95 or better. The initial rate for the 6.25 μM MPT

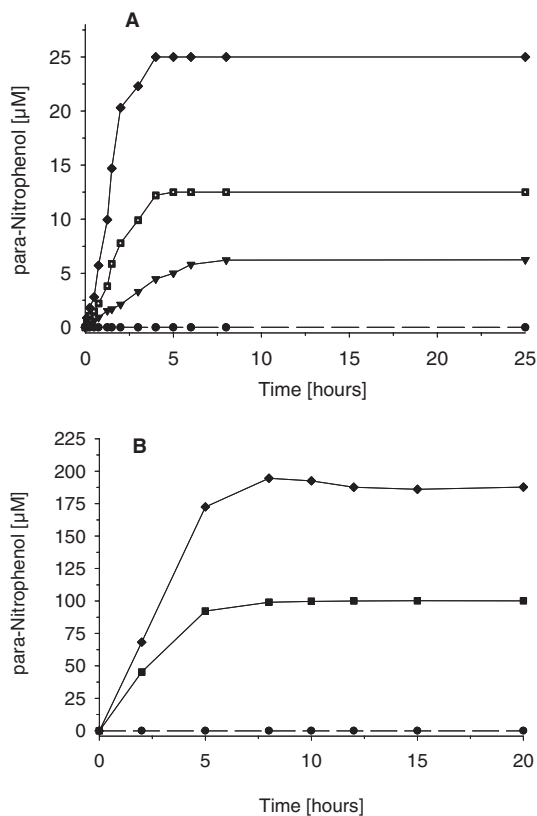


Figure 4. A) Catalytic activity of freeze-dried, crosslinked DPPE-DVBA/OPH vesicles shown by the enzymatic conversion of (∇) 6.25 μM , (\blacksquare) 12.5 μM , and (\blacklozenge) 25 μM MPT to PNP (formation was followed by absorbance measurements at room temperature), and (\bullet) control. B) Catalytic activity of freeze-dried, crosslinked DPPE-DVBA/OPH vesicles shown by the enzymatic conversion of (\blacksquare) 100 μM , and (\blacklozenge) 187 μM MPT to PNP; (\bullet) control.

through the crosslinked vesicle was $2.96 \times 10^{-10} \text{ M s}^{-1}$. Increasing the MPT concentration to 12.5 μM resulted in a 3.41-fold increase in the initial rate to $1.0 \times 10^{-9} \text{ M s}^{-1}$. At an initial concentration of MPT four times as large (now 25 μM), the initial rate was shown to be $2.6 \times 10^{-9} \text{ M s}^{-1}$, an 8.9-fold increase. In an attempt to maximize the hydrolyzing efficiency of OPH-containing vesicles, concentrations of 100 and 187.5 μM of MPT were tested. In Figure 4B, the data show that the hydrolysis of MPT was complete within five hours. Both data curves had a squared correlation coefficient of 0.989 or better. The initial rate of the diffusion of the 100 μM MPT through the crosslinked vesicle was $5.1 \times 10^{-9} \text{ M s}^{-1}$. Increasing the MPT concentration to 187.5 μM resulted in a 1.8-fold increase in the initial rate to $9.6 \times 10^{-9} \text{ M s}^{-1}$. In Figure 5, the effect of MPT concentration on the catalytic activity of freeze-dried, crosslinked DPPE-DVBA/OPH vesicles is shown as a plot of normalized catalytic activity versus MPT concentration. The data show that initially there is a sharp rise in the normalized catalytic activity for the hydrolysis of MPT to PNP for concentrations up to 25 μM . Thereafter, the normalized catalytic activity for the hydrolysis of MPT to PNP is slower, but still increasing. This result illustrates that hydrophobic molecules such as MPT can be transported across the bilayer membrane. Moreover, a linear response in the enzyme activity at higher MPT concentration indicates that the vesicles' bilayer membranes help control the amount of MPT available to interact with the encapsulated enzyme, thereby sustaining enzyme activity even in the overwhelming MPT concentration. It also serves to show that crosslinking the DPPE-DVBA headgroups increases the stabilization of the vesicle system without adversely affecting bilayer transport properties. In conventional vesicles, the structure is lost after freeze-drying. In this case, the structure was retained after crosslinking and freeze-drying. This increases the usefulness of the crosslinked vesicles as delivery vehicles that can be taken to the substrate. Furthermore, freeze-drying will increase the shelf life of the crosslinked vesicles.

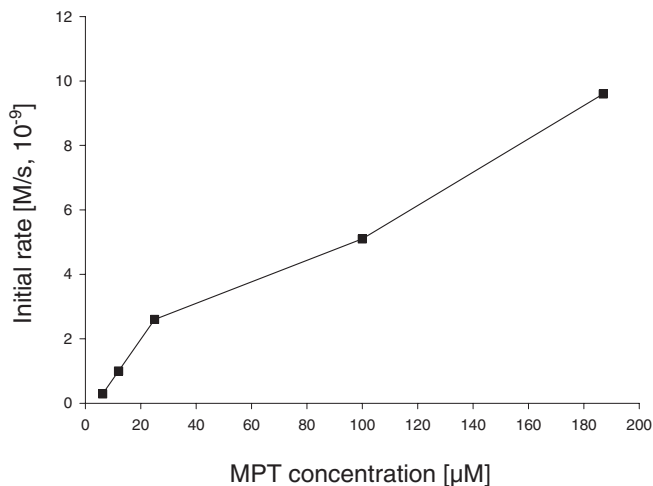


Figure 5. Effect of MPT concentration on the initial velocities of freeze-dried, crosslinked DPPE-DVBA/OPH vesicles at room temperature.

3. Conclusion

Phospholipid-based catalytic nanocapsules from DPPE-DVBA have been prepared. Efficient crosslinking polymerization by 5 min exposure to UV light makes the system technologically attractive for encapsulating fragile molecules such as enzymes, while retaining the bulk of the enzymes' catalytic activity. The crosslinked vesicles containing OPH or PTE catalyzed the hydrolysis of MPT, the rate of which was followed by monitoring the production of PNP. Specific activities for OPH in the presence of added CoCl_2 (a cofactor) in a buffer, and PTE without a cofactor, were found to be similar after crosslinking and freeze-drying the vesicles. This shows that the polymerization process does not deactivate either the OPH or PTE encapsulated in the crosslinked vesicles. Bilayers of crosslinked vesicles protected the enzyme entrapped within the vesicle core from deactivation resulting from UV exposure. The freeze-dried, crosslinked vesicles with encapsulated OPH were shown—by monitoring the production of PNP—to hydrolyze the hydrophobic molecule MPT. The crosslinked vesicles with OPAA encapsulated showed activity against DFP. Crosslinked vesicles with encapsulated enzymes may find applications in protecting individuals against exposure to organophosphate compounds on surfaces or in and around confined spaces.

4. Experimental

General: Unless stated otherwise, all solvents and reagents were purchased from Aldrich and used as received. For spectral characterization and analysis, an Agilent 8453 UV-vis spectrophotometer was used. The buffer used with OPH was a 10 mM BTP buffer (Sigma Aldrich, Milwaukee, WI) at pH 8.6 containing 100 μM CoCl_2 . The buffer used with OPAA was a 10 mM BTP buffer at pH 8.6 containing 100 μM MnCl_2 . OPH was received as a solid powder, OPH/trehalose (5:95 wt-%), from Dr. V. K. Rastogi of the US Army Chemical and Biological Defense Agency, Aberdeen Proving Ground, MD. Wild-type PTE was received from F. M. Raushel, and the experimental conditions have been previously published [1]. OPAA was received as a purified protein from T. C. Cheng of the US Army Chemical and Biological Defense Agency, Aberdeen Proving Ground, MD. DPPE-DVBA was prepared as previously described [1].

Preparation and Characterization of Vesicles: DPPE-DVBA (5 mg) was dissolved in chloroform (0.5 mL). A thin lipid film was coated onto the walls of the tube by removing solvent under a gentle stream of nitrogen, followed by thoroughly drying the film under a high vacuum. Typically, the lipid concentration in the vesicles was 5 mg mL^{-1} . Thin lipid films were hydrated in BTP-Co buffer (1 mL; 100 μM) at pH 8.6 for experiments using OPH. For OPAA, BTP- MnCl_2 buffer (100 μM) at pH 8.6 was used. For experiments using PTE, CHES buffer (10 mM) was used for hydration at pH 8.6. Lipid dispersions were hydrated by heating at 55 °C for 1 h and then dispersed in the medium by intermittent vortex-mixing followed by sonication at 55 °C using a Branson Sonifier model 450. A cup-horn device equipped with water-intake and -outlet connections was used for sonicating the sample. In all cases, 1 h sonication at 50 % power and 80 % duty cycle produced dispersions of constant turbidity, leading to the formation of uniform-sized vesicles. The dispersions were quickly frozen in a dry-ice/ethanol bath, and subjected to high vacuum using a Labconco freeze-drying system (model freezezone 4.5).

TEM images of the vesicles were acquired using the same protocol and instrumentation previously described [1]. Images of the vesicles

were acquired using a Zeiss EM-10 transmission electron microscope equipped with Spot Insight QE digital camera (model 4.2) at 60 kV. Typically, a drop of vesicle suspension was placed on 200 mesh copper formvar/carbon grid. Vesicles on the grid were stained by placing a drop of 1 % uranyl acetate in water followed by removing excess solution by wicking it with a piece of filter paper.

Encapsulating Enzymes in Vesicles: OPH, PTE, and OPAA were used for encapsulation experiments. To encapsulate OPH, a BTP- CoCl_2 buffer (2 mL; 100 μM) was used. To encapsulate PTE, CHES buffer (2 mL; 10 mM) was used. To encapsulate OPAA, BTP- MnCl_2 buffer (2 mL; 100 μM) was used. Freeze-dried, non-polymerized vesicles were used for the enzyme encapsulation experiments. The OPH concentration was either 100 $\mu\text{g mL}^{-1}$ or 300 $\mu\text{g mL}^{-1}$. The PTE concentration was 100 $\mu\text{g mL}^{-1}$. For optimization of encapsulation efficiencies, additional enzyme concentrations were used; [OPH] = 600 and 200 $\mu\text{g mL}^{-1}$ and [PTE] = 200 $\mu\text{g mL}^{-1}$. The OPAA concentration used was 100 $\mu\text{g mL}^{-1}$. All enzyme solutions were in buffer (2 mL; pH 8.6) and added to, powdered, freeze-dried, non-polymerized vesicles (4 mg). The vesicles were slowly hydrated at 50 °C for 3 h with occasional vortex agitation to give a translucent vesicle dispersion. Sephadex gel (4.0 g; G 50-150) was soaked in deionized water, degassed, and poured into a glass column to form a column of gel (20 cm \times 1 cm). The Sephadex column was primed with buffer before loading the vesicle/enzyme dispersion. Exogenous enzyme was separated from the vesicle-encapsulated enzyme by gel filtration. A 0.5 mL volume was collected in each fraction and the presence of vesicles and enzyme was confirmed by monitoring for OPH at 263 nm (PTE at 279 nm and OPAA at 290 nm) and vesicles at 400 nm. After combining the vesicle-encapsulated enzyme fractions, one half was set aside for characterization of the vesicle integrity and the other half was crosslinked.

Polymerization of Vesicles: All polymerization experiments were carried out at room temperature using the same conditions and protocols as described previously [1]. The enzyme-containing vesicles were stabilized by crosslinking using a combination of the water-soluble radical initiator AAPD and subsequent UV irradiation at 254 nm for 5 min.

Enzymatic Activity of OPH-, PTE-, and OPAA-Containing Vesicles: From the combined fractions of the non-polymerized, enzyme-containing vesicle fractions collected from gel filtration, 100 μL aliquots were withdrawn and placed in a quartz UV cuvette. An MPT solution (500 μL ; 100 μM) in 25 % aqueous methanol was added to the vesicle dispersion. The contents in the cuvette were thoroughly mixed and the generation of PNP was monitored for 120 s by continuously recording the absorbance at 405 nm. The initial rate was measured based on the production of PNP with time. A similar protocol was used for determining the enzyme activity in the crosslinked vesicles. As a control, the activity of enzyme dissolved in buffer was determined before and after exposure to UV irradiation for 5 min.

Hydrolysis of MPT in Freeze-Dried, Crosslinked DPPE-DVBA Vesicles: Only vesicles with the enzyme OPH were used in this study. The crosslinked DPPE-DVBA and DPPE-DVBA/OPH vesicles used were formed using the above procedures. Control experiments were prepared by the following methods. An aliquot of MPT solution (500 μL ; 26 μM) in 25 % aqueous methanol was added to BTP- CoCl_2 buffer (1.5 mL; 100 μM) and stirred until homogenous. The second control experiment was prepared by adding freeze-dried, empty crosslinked vesicles (2 mg) to BTP- CoCl_2 buffer (1.5 mL; 100 μM) and vortex-mixing the solution at room temperature until a translucent suspension was obtained. An aliquot of MPT solution (500 μL ; 26 μM) in 15 % aqueous methanol was added and the contents stirred thoroughly. The control experiments were followed by UV-vis spectrophotometry at 405 nm by monitoring the production of PNP. The hydrolysis experiment was prepared by adding freeze-dried DPPE-DVBA/OPH vesicles (2 mg) to BTP- CoCl_2 buffer (1.5 mL; 100 μM). After vortex-mixing at room temperature to obtain a translucent suspension, an aliquot of MPT solution (500 μL ; 100 μM) in 15 % aqueous methanol was added. The hydrolysis of MPT was monitored by following the production of PNP at 405 nm.

Received: April 8, 2004
Final version: July 26, 2004

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