Interfacial catalysis by phospholipases at conjugated lipid vesicles: colorimetric detection and NMR spectroscopy

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Background: Self-assembled conjugated polymers are rapidly finding biological and biotechnological applications. This work describes a synthetic membrane system based on self-assembled polydiacetylenes, which are responsive to the enzymatic activity of phospholipases – a ubiquitous class of enzymes that catalyze the hydrolysis of phospholipid molecules embedded in cell membranes.

Results: We show that phospholipases are active at bilayer vesicles composed of the natural enzyme substrate, dimyristoylphosphatidylcholine (DMPC), and a synthetic π-conjugated polymerized lipid based on polydiacetylene (PDA). In addition, the enzymatic reaction induces an optical transition in the surrounding PDA matrix, visible to the naked eye. Nuclear magnetic resonance spectroscopy confirms the occurrence of enzymatic catalysis and reveals the fate of the cleavage products.

Conclusions: The results indicate that the structural and color changes of the PDA matrix are directly related to interfacial catalysis by phospholipase. This novel biocatalytic method of inducing optical transitions in conjugated polymers might lead to new approaches towards rapidly screening new enzyme inhibitor compounds.

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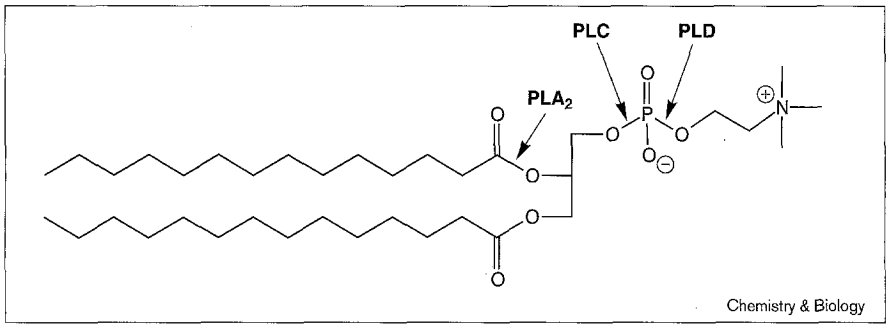
Introduction

Self-organized π-conjugated polymers, such as polydiacetylene (PDA), polythiophene and polypyrrole, offer a variety of possibilities in the development of novel, flexible electronic devices, such as photodiodes [1], light-emitting devices (LEDs) [2] or organic transistors [3]. These materials, which are often referred to as 'organic semiconductors' or 'synthetic metals', display a remarkable array of color transitions arising from thermal changes (thermochromism), [4] mechanical stress (mechanochromism) [5] or ion binding (ionochromism) [4,6]. The color transitions in these materials have been ascribed to a change in the effective conjugation length of the delocalized π-conjugated polymer backbone [7]. The biological applications of conjugated polymers are only now beginning to be exploited. In particular, the chromic transitions can be utilized for the detection of biological macromolecules (biochromism) [8–10]. For example, it has been shown that PDA vesicles and thin films can be used to detect the presence of viruses or toxins through a blue-to-red color transition [8,11–14]. In these earlier studies, binding a 1000 Å virus particle to a carbohydrate-modified PDA vesicle induced the blue-to-red transition. In order to detect protein molecules of lower molecular weight (e.g. 400 kDa or less), however, the PDA vesicle or film system required either the use of specific 'dopant' lipids [12], or that the diacetylene unit be positioned closer to the interfacial region [13]. Simply binding a low molecular weight enzyme to the vesicle system as described here is therefore not anticipated to induce the color transition.

In this report, we show that chemical changes at the PDA vesicle interface, induced by enzymatic catalysis, does, in fact, lead to colorimetric responses. Specifically, the color transitions of PDA matrices act as optical 'reporters' for the interfacial catalysis carried out by three lipolytic enzymes: phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD). The study of PDA systems in conjunction with biological processes is actually a two-pronged approach; besides the potential applications of PDA materials to biological recognition and biocatalysis, one can also develop a better understanding of the molecular nature of the optical transitions occurring within the PDA matrix, for a variety of biological stimuli.

Numerous enzymatic reactions occur at interfaces of biomembranes, or similar amphiphilic aggregates in aqueous solutions, and are usually referred to as interfacial catalytic processes. Phospholipases, in particular, are an important class of interfacial enzymes. Although these enzymes are, in general, water soluble, they catalyze the hydrolysis of phospholipid molecules at the water–lipid interface of cellular

Figure 1



The structure of dimyristoylphosphatidylcholine (DMPC), indicating the points of cleavage by the enzymes PLA_2, PLC and PLD.

membranes. Phospholipases are involved in diverse biochemical processes, such as fat digestion, lipid metabolism and the regulation of signal transduction [15]. Considerable interest in PLA_2 stems from its direct role in the arachidonate cascade, leading to the formation of a variety of regulatory molecules such as prostaglandins and leukotrienes [15,16]. Similarly, PLC is believed to play a key role in the generation of second messengers in mammalian cells, which also might result in, for example, an increased production of prostaglandins [15]— compounds implicated in the development of disease conditions such as rheumatoid arthritis and pancreatitis [15,16]. Phosphatidic acid (PA), the hydrolysis product of PLD, is also involved in a number of cell-signaling processes. Many functions for PA have been proposed, including activation of protein kinases, other phospholipases and neutrophils [17]. The negatively charged enzymatic lipid product of PLD is also thought to facilitate vesicular transport through the attraction of membrane proteins [18].

Phospholipases have also been identified as toxins. For example, they are believed to be among the most toxic elements of snake venom [19]. The venom toxins could be PLA_2 enzymes themselves, or exhibit 'PLA_2-like' activity at specific membrane interfaces such as pre-synaptic termini. The phospholipases found in snake venom often share a high degree of structural homology with their mammalian counterparts [19].

A large body of work has been devoted to understanding phospholipase activities. Several studies have focused on characterizing phospholipase catalysis at model membrane interfaces, such as liposomes [20–22], polymerized vesicles [23–25] or Langmuir monolayers [23,26,27]. In this paper, we describe a new type of model membrane system based on vesicles composed of optically responsive, conjugated PDA amphiphiles surrounding the natural, unlabeled enzyme substrate, dimyristoylphosphatidylcholine (DMPC). We show that this model system not only facilitates interfacial enzymatic catalysis of DMPC hydrolysis by phospholipases, but also provides a sensitive colorimetric response that is indicative of the hydrolysis reaction.

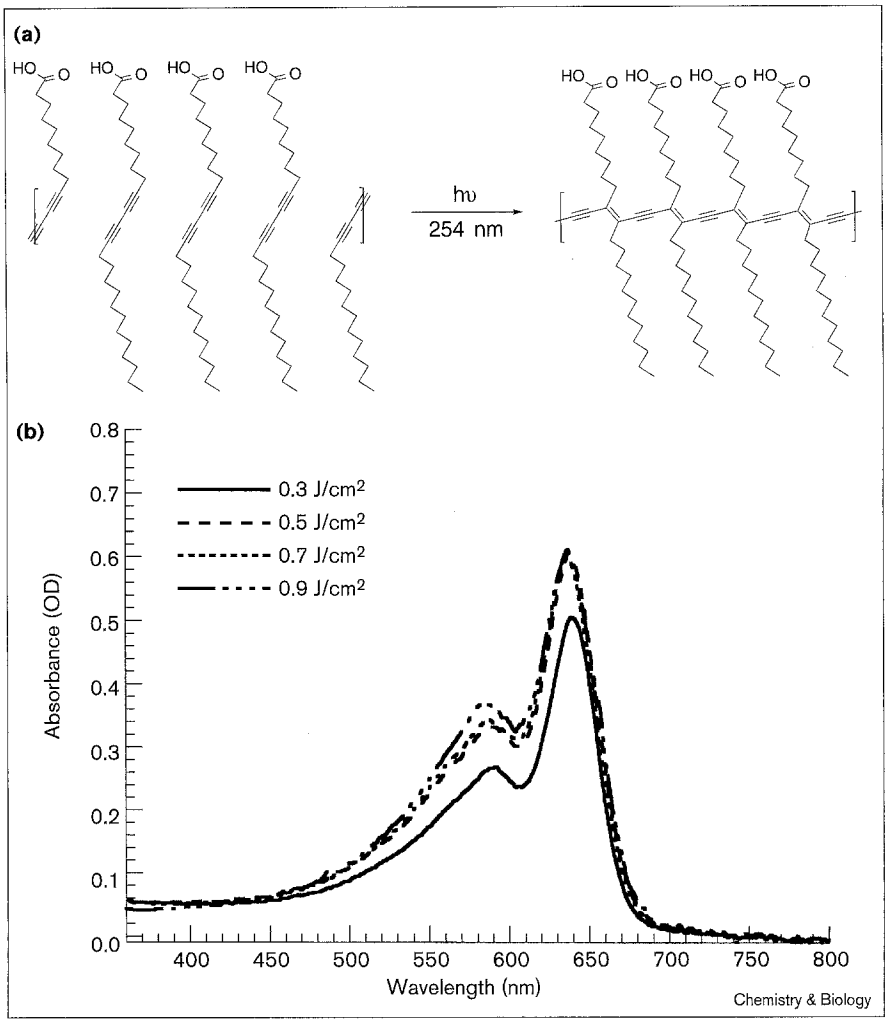
Each of the three enzymes discussed in this study hydrolyzes a different bond of the substrate (Figure 1). PLA_2 acts closer to the lipid tail, whereas PLC and PLD act specifically at the hydrophilic headgroup region. Although the hydrolysis and reaction products are different in the three enzymatic processes, the conjugated PDA system exhibits colorimetric responses associated with enzymatic activity for all three biomolecules. The results indicate that color changes of the PDA matrix can be induced through interfacial processes that have not been described previously. It is also shown that nuclear magnetic resonance (NMR) spectroscopy can be used to verify independently the occurrence of interfacial catalysis and for tracking the structural and dynamical properties of the reaction products. This work suggests that mixed vesicles composed of enzyme substrates and polydiacetylene lipids offer the possibility of designing colorimetric biosensors for the detection of toxins exhibiting enzymatic activities and for screening compound libraries for inhibitory properties.

Results and discussion

Mixed polydiacetylene vesicles

The vesicles developed for this study are composed of DMPC, a natural substrate of the phospholipases, inserted into a matrix of the optically responsive PDA polymer. Upon UV (254 nm) radiation, diacetylenic lipids undergo solid-state polymerization, controlled by the packing of the monomers. The resulting polymer is highly conjugated due to the delocalized ene-yne π electronic system (Figure 2a). The time course of the polymerization reaction can be followed using visible absorption spectroscopy in the region of 450–650 nm (Figure 2b). The visible absorption arises from the conjugated ene-yne system that comprises the polymer backbone. (The monomer absorption occurs at wavelengths less than 300 nm.) The absorption intensity increases with the UV irradiation time and nearly saturates after a total energy dose of 0.5 J/cm^2, whereupon the vesicle solution takes on a deep blue color. The main excitonic absorption at ~620 nm and the associated 580 nm vibronic band correspond to the blue appearance of the vesicle solution. The results indicate that the high percentage of

Figure 2



(a) Polymerization reaction of diacetylenic lipids forming the conjugated polydiacetylene (PDA) backbone. **(b)** UV–visible spectra of DMPC/PDA as a function of irradiation dose. The vesicles are composed of 40% DMPC and 60% tricosadiynoic acid (TRCDA).

nonpolymerizable DMPC lipids incorporated into the PDA vesicles does not significantly interfere with the polymerization process.

As a result of the nature of the solid-state polymerization reaction, the appearance of the colored polymer provides a sensitive and simple test of molecular order in the self-assembled nanostructure. In general, smaller and 'looser' structures such as micelles do not give rise to conjugated polymers. The formation of vesicles in sonicated samples of amphiphilic diacetylenes has been demonstrated previously using electron microscopy [28,29]. In the current study, vesicles composed of 40% DMPC/PDA are approximately 100–200 nm in diameter, as measured using transmission electron microscopy (TEM).

Vesicle color changes induced by interfacial catalysis

Phospholipases act at different positions in the phospholipid molecule. The activity of phospholipases can be divided into two broad categories: the acyl hydrolases and the phosphodiesterases. The former group contains phospholipase A_1 (PLA_1) and PLA_2, which hydrolyze the 1- and 2-acyl ester bond of glycerophospholipids, respectively, yielding the corresponding fatty acids and lysophospholipids. The phosphodiesterases include PLC and PLD, which attack the phosphate ester moiety within the lipid hydrophilic headgroup. Figure 1 summarizes the hydrolysis sites within the substrate for each of the three phospholipases investigated in this report.

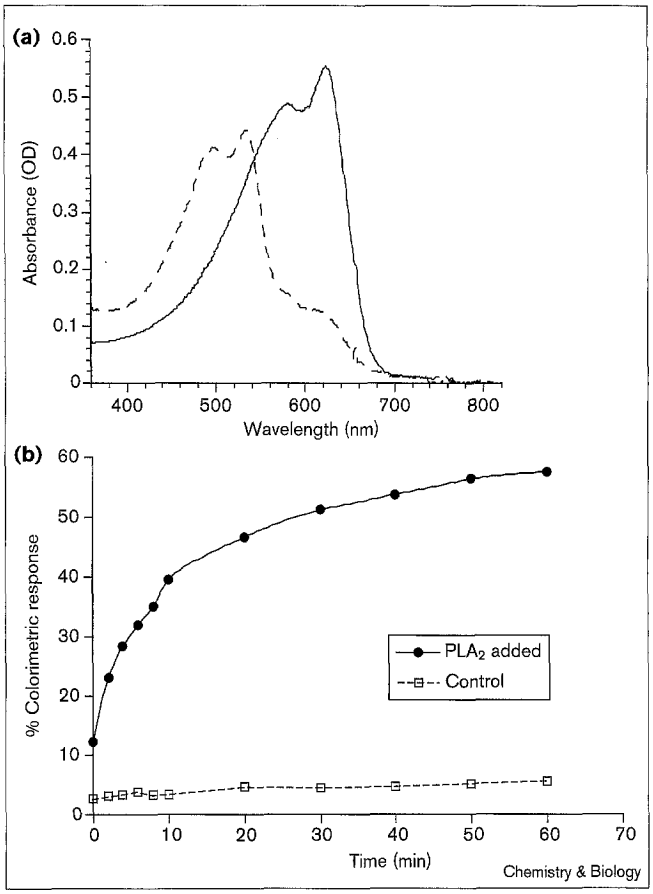
Various methods have been developed to assay phospholipase activity. Substrate recognition and product identification can be problematic using conventional assays because of the amphiphilic nature of the enzyme substrate. For example, PLA_2 is capable of hydrolyzing freely water-soluble lipids, but its activity is significantly reduced in these systems compared to organized assemblies such as micelles or vesicles [30,31]. A common method for assaying phospholipases is through titration, which measures the proton liberated during hydrolysis [32]. Radioactive-tracer

techniques [33] are often used to detect less active cellular phospholipases. Although sensitive, these methods are generally laborious and also incur significant costs because they involve the use of radiolabeled substrates. Colorimetric assays using thioester phospholipid derivatives as substrates have also been used [34]. This technique is quite convenient, but it is not certain that the model compounds used are suitable substrates for all phospholipases, compared to the natural oxy-acyl ester substrates.

The results obtained in this study demonstrate that insertion of the natural substrate of phospholipases into an optically responsive matrix, which is sensitive to interfacial catalysis, alleviates many of the concerns mentioned above. Figure 3 indicates the colorimetric response of mixed DMPC/PDA vesicles to enzyme activity. Figure 3a shows the UV–visible spectra of vesicles containing 40% DMPC/PDA prior to, and after, the addition of PLA_2. As the enzyme is added, the color of the vesicle solution clearly changes from blue to red. The absorption maximum at 620 nm gradually decreases, whereas the 540 nm and 490 nm peaks increase in intensity. The latter wavelengths correspond to the main excitonic and vibrational bands, respectively, of the red-phase polymer. The time course of the colorimetric reaction of DMPC/PDA vesicles with PLA_2 is shown in Figure 3b. The results indicate that the surrounding PDA matrix is responsive to the effects of hydrolysis of the 2-acyl ester bond of the DMPC lipid that is embedded in it. Previous data have indicated that the color change does not occur in the absence of DMPC in vesicles [10]. In addition, PLA_2 activity was measured independently using a labeled lipid analog incorporated into the PDA matrix, allowing simultaneous measurement of product formation and colorimetric response of the vesicles [10]. Finally, we have also demonstrated that the catalytically induced color change in mixed DMPC/PDA vesicles can be suppressed by the addition of known inhibitors of PLA_2 [10]. The mixed vesicle system described here might therefore be applicable to high-throughput screening of novel enzyme inhibitor compounds. Using a 96-well format, effective drug candidates can be identified by detecting vesicle solutions maintaining their blue color, even after addition of the enzymes.

Phospholipases that hydrolyze the headgroup of DMPC similarly induce color changes in the DMPC/PDA vesicles. Figure 4a and 4b shows the time courses of colorimetric response for the enzymes PLC and PLD, respectively. The difference in the progression of the color changes between the two enzymes might reflect the fact that the hydrolysis rates of the enzymes are not identical. The different rates of colorimetric response might also be associated with molecular rearrangements of the catalysis products within the vesicles. The NMR data presented below support this description.

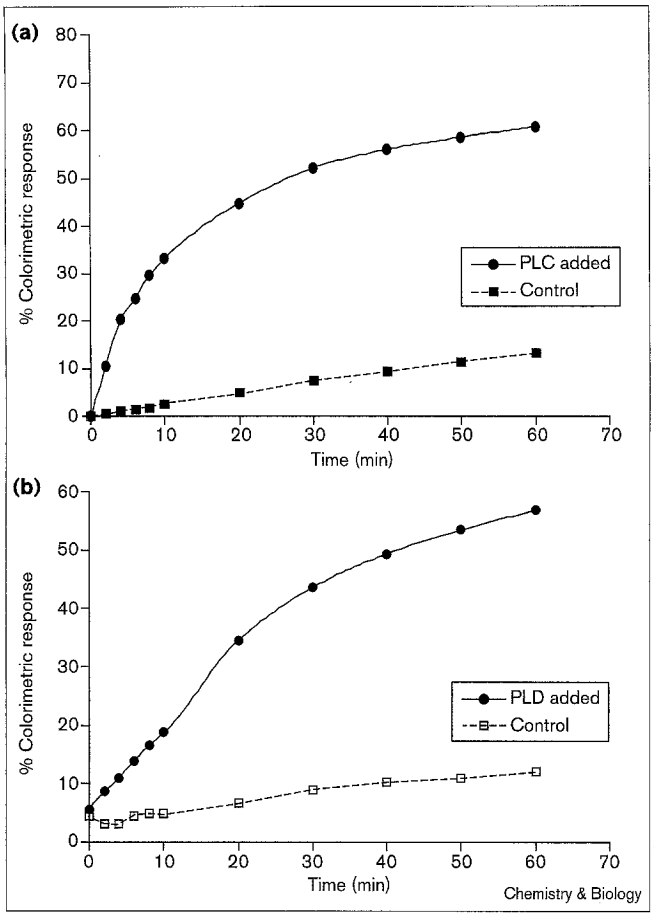
Figure 3



(a) UV–visible spectra of DMPC/PDA vesicles before (solid line) and after (dashed line) addition of PLA_2. **(b)** Colorimetric response curve of DMPC/PDA vesicles after the addition of PLA_2. A colorimetric response of 15% is easily seen with the naked eye. Control experiments contain all the components except the enzyme.

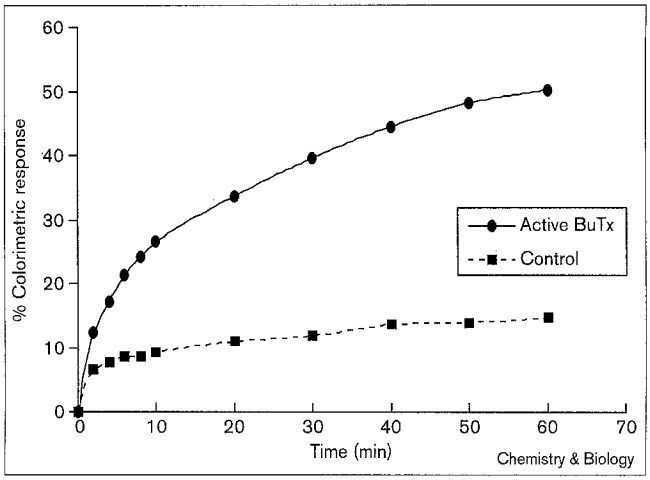
Vesicles of DMPC/PDA can also be used for the detection of toxins that exhibit enzyme-like activity. For example, β-bungarotoxin (BuTx), isolated from snake venom, acts specifically at the presynaptic motor nerve termini to alter neurotransmitter release and the distribution of aminophospholipids in the inner- and outer-membrane leaflets [35]. This toxin is also of significance because of its suspected use as a potential biological warfare agent, and methods that allow for its rapid detection are sought. Indeed, the mixed vesicles composed of DMPC/PDA are responsive to the presence of BuTx, as shown in Figure 5, which depicts the color changes of DMPC/PDA vesicles induced by BuTx. Although the maximum response in this system is achieved only after ~50 minutes, the color change of the solution is easily visualized after 10 minutes (%CR ~25; where % CR is % conversion to red; see the Materials and methods section for details). BuTx has previously been shown to induce the fusion of small unilamellar liposomes [36]. The fusogenic effects of BuTx are strongly related to the known phospholipase-like activity of the toxin.

Figure 4



Colorimetric response curves for DMPC/PDA vesicles upon addition of **(a)** PLC and **(b)** PLD. Control experiments contain all the components except the enzyme.

Figure 5



Colorimetric response curves of DMPC/PDA vesicles upon addition of β-bungarotoxin (BuTx). Control experiments contain all the components except the toxin.

NMR analysis of lipid hydrolysis

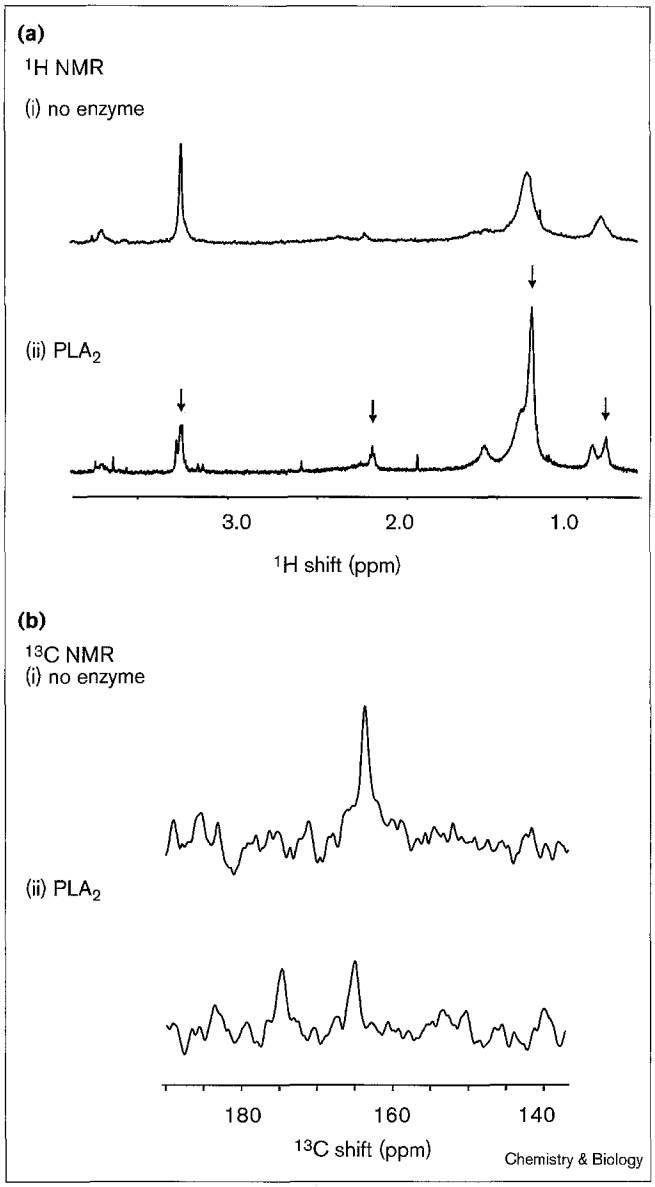
NMR analysis of mixed DMPC/PDA vesicles hydrolyzed with phospholipases provides molecular information on the interfacial catalysis and the fate of the reaction products. Parallel measurements of the colorimetric transitions of the vesicles, and acquisition of ^{1}H, ^{13}C, and ^{31}P NMR spectra yield valuable data on the relationships between the enzymatic reactions and the color changes occurring at the vesicle polymeric matrix.

Figures 6 and 7 feature NMR spectra of mixed DMPC/PDA vesicles digested with PLA_2. The NMR data clearly show that addition of the enzyme induces structural changes within the vesicle assembly. Specifically, the ^{1}H spectra in Figure 6a indicate the appearance of two separate molecular components in the aqueous solution following the addition of the enzyme. Distinct ^{1}H signals emerge following the enzymatic reaction and are assigned to CH_3 protons at around 0.9 ppm, CH_2 of the alkyl chains at around 1.4 ppm, and the methylene CH_2COOR at around 2.3 ppm. In addition, sharp ^{1}H resonances appear in the spectral region corresponding to the phosphatidylcholine headgroup at around 3.3 ppm. Sharpening of resonances is similarly observed for the α and β CH_2 protons of the carboxylic moiety following the enzymatic hydrolysis (Figure 6a,ii). The appearance of distinct ^{1}H resonances associated with enzymatic cleavage products is directly related to the incipient color changes within the vesicle assembly. For example, the vesicle solution giving rise to the spectrum shown in Figure 6a,ii exhibits a purple color indicating an approximately 50% transition from the blue phase to the red phase. Furthermore, the change in intensities of the distinct NMR peaks ascribed to the reaction products can be quantitatively correlated with the optical response of the vesicles (data not shown).

The generation of two distinct molecular species upon cleavage of DMPC by PLA_2 is confirmed by the ^{13}C NMR data shown in Figure 6b, which were acquired using mixed vesicles containing DMPC molecules isotopically labeled with ^{13}C at the two carbonyl positions. A single ^{13}C resonance at around 160 ppm is observed for the undigested vesicles (Figure 6b,i), whereas addition of the enzyme gives rise to two downfield peaks at around 165 ppm for the ester and 175 ppm for the acid, respectively (Figure 6b,ii).

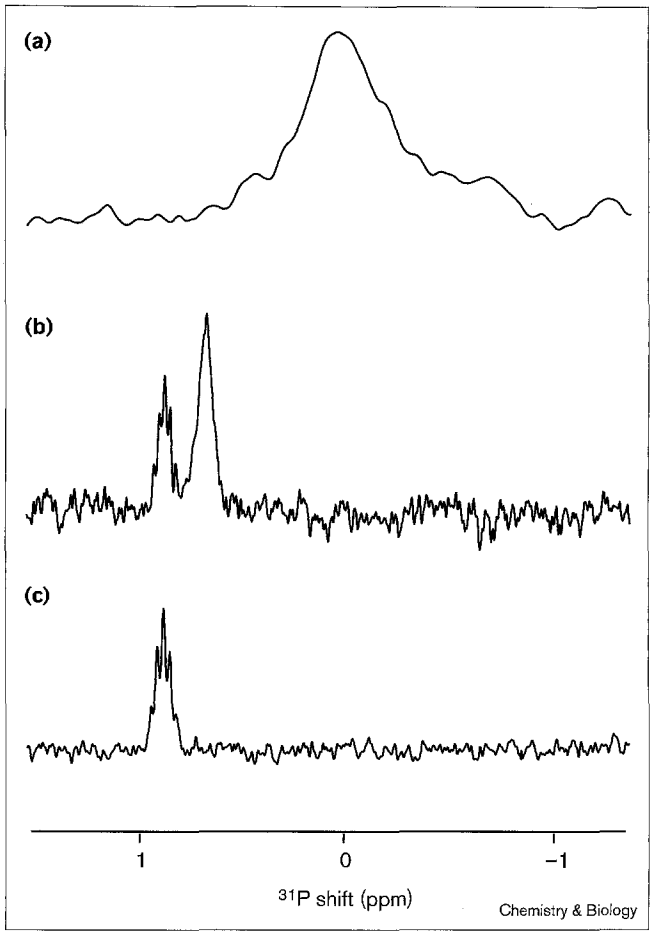
The appearance of two molecular components upon addition of PLA_2 is consistent with the catalytic cleavage of the ester bond of DMPC by the enzyme. Furthermore, the NMR data suggest that the hydrolysis products are released from the vesicle matrix into the solution, yielding the narrow ^{1}H and ^{13}C resonances observed in Figure 6a,ii and Figure 6b,ii, respectively. Strong support for this hypothesis is provided by the ^{31}P NMR data shown in Figure 7. Figure 7a features a ^{31}P spectrum of

Figure 6



NMR spectra of DMPC/PDA vesicles (0.5 μmol total lipid) mixed with PLA_2 (0.7 nmol): **(a)** ^{1}H; **(b)** ^{13}C. The arrows indicate new peaks associated with soluble reaction products. See text for details.

Figure 7

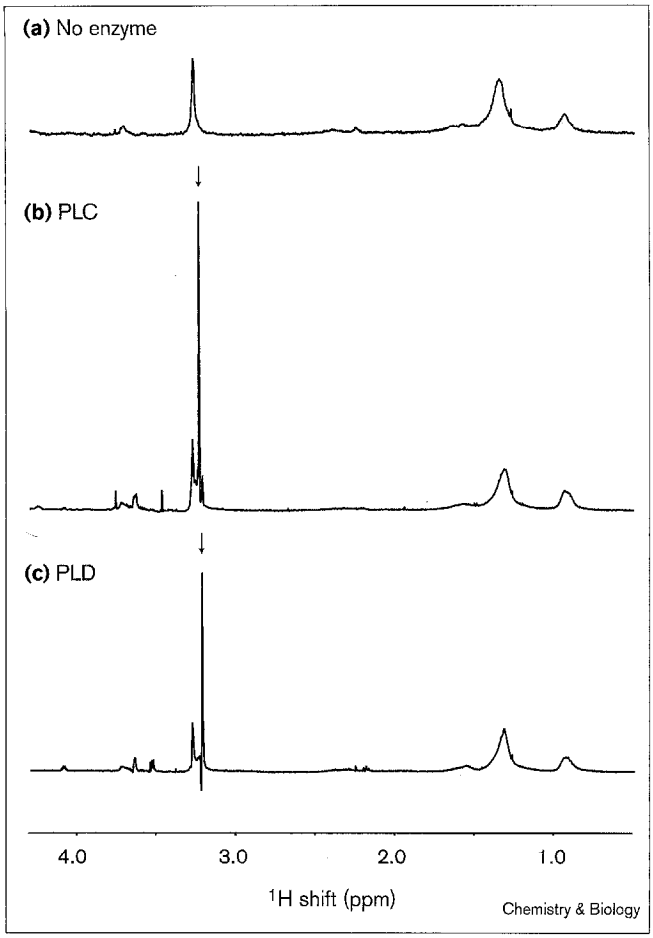


^{31}P NMR spectra of DMPC/PDA vesicles. **(a)** Intact vesicles; **(b)** vesicles mixed with 0.7 nmol of PLA_2; **(c)** vesicle–enzyme mixture of (b) to which 31 mmol $CaCl_2$ has been added (resulting in precipitation of the vesicles).

intact DMPC/PDA vesicles. The relatively broad ^{31}P signal at around 0 ppm indicates constrained mobility of DMPC within the polymerized vesicles. Addition of PLA_2 gives rise to two distinct ^{31}P signals (Figure 7b). The downfield quintuplet at around 0.9 ppm is most likely due to soluble lysophospholipid, in which the the splitting of the ^{31}P resonance by the glycerophosphocholine protons is resolved because of the rapid molecular tumbling. The upfield ^{31}P resonance at around 0.65 ppm in Figure 7b, on the other hand, probably corresponds to uncleaved DMPC molecules. This peak, however, is significantly sharper than the parallel ^{31}P signal observed for the DMPC within the mixed vesicles untreated with PLA_2 (Figure 7a). The sharp ^{31}P resonance is most likely due to highly mobile undigested DMPC molecules associated with 'holes' formed within the vesicles following the enzymatic catalysis and the release of the hydrolysis products. Evidence for the ejection of phospholipase-cleavage products from membrane environments has been reported previously in physiological and synthetic systems [26].

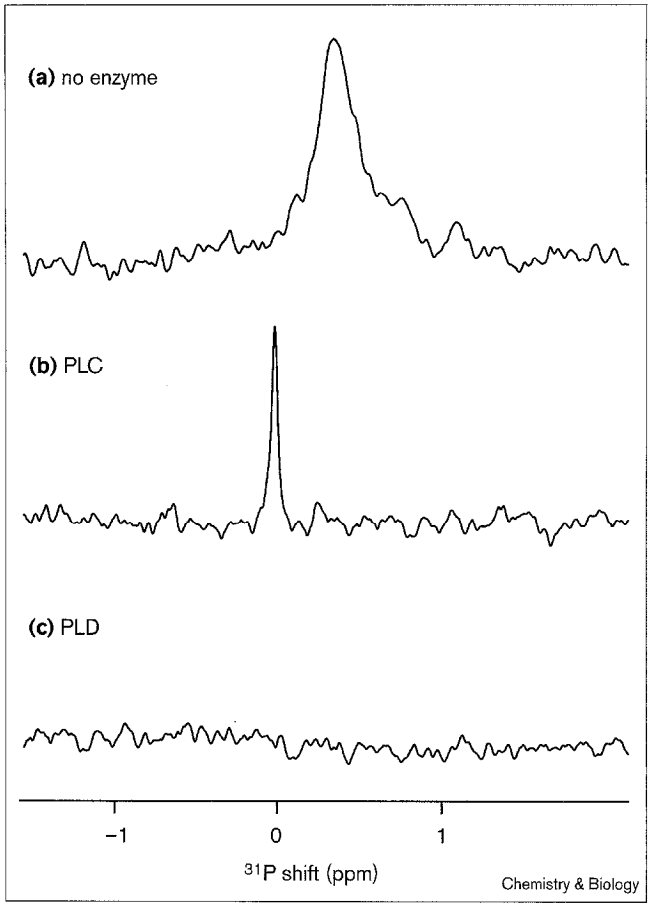
In order to verify the assignments of the ^{31}P resonances in Figure 7b to soluble and vesicle-incorporated components, we performed an experiment in which agglutination of the vesicles was induced, and a ^{31}P spectrum of the supernatant was acquired (Figure 7c). Addition of about 31 mmol excess $CaCl_2$ salt results in aggregation and precipitation of the vesicles. Consistent with the spectral assignments outlined above, Figure 7c shows that the upfield ^{31}P signal at 0.65 ppm, which has been assigned to vesicle-incorporated DMPC, disappears because of the

Figure 8



^{1}H NMR spectra of: **(a)** intact DMPC/PDA vesicles; **(b)** DMPC/PDA vesicles (0.5 μmol) mixed with PLC (1 nmol); **(c)** DMPC/PDA vesicles (0.5 μmol) mixed with PLD (1 nmol). The arrows indicate the soluble choline residue. See text for details.

Figure 9



^{31}P NMR spectra of: **(a)** intact DMPC/PDA vesicles; **(b)** DMPC/PDA vesicles (0.5 μmol) mixed with PLC (1 nmol); **(c)** DMPC/PDA vesicles (0.5 μmol) mixed with PLD (1 nmol).

aggregation. The ^{31}P quintuplet at around 0.9 ppm, which corresponds to soluble lysophospholipid, is not affected by agglutination of the vesicles, however.

^{1}H NMR experiments shown in Figure 8 confirm the occurrence of interfacial enzymatic catalysis in solutions containing DMPC/PDA vesicles and the enzymes PLC and PLD. A sharp, intense ^{1}H resonance corresponding to the soluble choline group is observed, as expected, in both vesicle–enzyme mixtures (Figure 8b and Figure 8c, respectively). The sharpness of the peaks allows resolution of the singlet resulting from the trimethyl ammonium group (3.2 ppm) and the methylene groups (3.3 ppm) of the choline. In contrast to the ^{1}H NMR results obtained for PLA_2 (Figure 6), however, no new spectral features are detected in the methyl and methylene regions around 1 ppm (Figure 8). This result indicates that the lipid backbone of DMPC is not affected strongly by either PLC or PLD, and remains incorporated within the PDA matrix.

The ^{31}P data shown in Figure 9 provide additional information on the enzyme reactions and the fate of the cleavage products. Mixing PLC with DMPC/PDA vesicles yields a sharp ^{31}P signal corresponding to soluble phosphocholine (Figure 9b). When PLD is added to a solution of DMPC/PDA vesicles, however, the ^{31}P resonance completely disappears (Figure 9c). The disappearance of the ^{31}P signal is most likely due to constriction of the mobility of the phosphate residues at the vesicle surface, which would induce extreme broadening of the NMR resonance. Immobilization of the phosphate group might be associated with salt-bridging through ionic interactions between the negatively charged phosphate residues and the Ca^{2+} dissolved in the buffer solution.

The ^{1}H, ^{13}C and ^{31}P NMR data shown in Figures 6–9 indicate that significant spectral and structural changes are already observed upon addition of even a relatively small quantity (10 ng) of the enzymes examined. These results are consistent with the catalytic properties of phospholipases.

Transmission electron microscopy

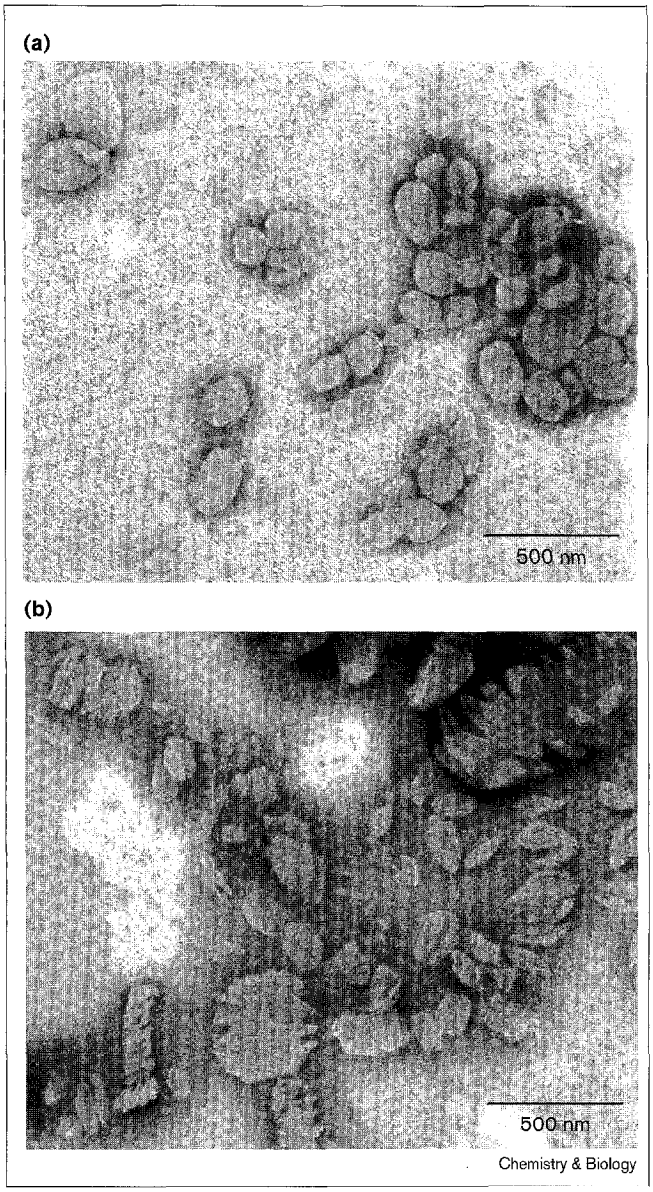
TEM images of the DMPC/PDA vesicles indicate that enzymatic hydrolysis involving all three phospholipases results in large-scale morphological and structural changes of the vesicles. Figure 10 shows the representative TEM images of DMPC/PDA vesicles before and after mixing with PLC. TEM images of the vesicles reacting with PLA_2 and PLD were very similar to the one shown in Figure 10b. The PLC-treated vesicles appear significantly more cracked and pitted than the unhydrolyzed mixed vesicles (Figure 10a). The 'pits' shown in Figure 10b might be related to the considerable structural rearrangement within the vesicles, described below. Significantly, the TEM image of the enzyme-digested DMPC/PDA vesicles clearly indicates that the color change of the polydiacetylene matrix lipids is associated with structural modifications undergone by the vesicles. By combining the TEM results with the NMR spectroscopic data, it is possible to construct molecular-level analysis of the optical and structural changes of the conjugated polymers.

Perturbations of DMPC/PDA vesicles induced by interfacial catalysis

We have shown that colorimetric transitions of the conjugated polymer vesicles are directly related to the activities of phospholipases, and that the mixed vesicles provide an optical 'probe' for investigating the effects of lipolytic enzymes on membrane microstructures. In general, it has been determined previously that color transitions of PDA-based materials (e.g. crystals and films) are affected by changes in the conformation, packing and ordering of the pendant alkyl side-groups [7,37–39]. Local changes in membrane organization, packing order, or microenvironments, could therefore directly induce color changes within the polymerized vesicles. Detailed NMR and TEM analyses indicate that significant changes in the morphology and packaging of the liposomes are associated with the observed color transitions. In particular, the data have provided evidence that contraction or distortion of liposome structure, which would affect the conjugation length of the PDA system, is directly correlated with both thermochromic as well as biochromic transitions (R.J. and D.C., unpublished observations).

Specifically, in the mixed DMPC/PDA systems investigated here, the perturbation of the conjugated matrix is induced through the enzymatic cleavage of chemical bonds in the substrate molecules. The NMR data have clearly shown, for example, that molecular rearrangements occur within the vesicles following the enzymatic reactions. Although the three phospholipases examined cleave the substrate at different locations, the substrates are structurally modified by the enzyme action in all cases, and the surrounding membrane is perturbed, leading to the color changes.

Figure 10



Representative TEM image of DMPC/PDA vesicles **(a)** before and **(b)** after exposure to PLC. Images similar to that shown in (b) were obtained for DMPC/PDA vesicles treated with PLA_2 or PLD. The vesicles were stained with phosphotungstic acid.

In the case of PLA_2 catalysis, it is clear from the NMR data that the hydrolysis products (fatty acid and lysolipid) leave the membrane matrix. Previous studies reveal that such 'uncorking' of vesicles leads to leakage of fluorescent dyes previously trapped and quenched in the vesicle interior [23,40]. In the case of the PDA vesicles studied here, the release of the natural lipids results in a significant change in lipid density and the appearance of pits and cracks. Changes in lipid packing were previously shown to result in color changes for PDA monolayer films at the air–water interface [41,42].

Unlike PLA_2, PLC cleaves at the head-group region of DMPC (Figure 1). The PLC used in this study is derived from *Bacillus cereus*, and is similar to mammalian PLC in both function and immunological properties [43,44]. The ^1H and ^{31}P data discussed above confirm that the product of PLC hydrolysis, 1,2-diacylglycerol (DG), is indeed formed within the vesicles. DG is generally known to destabilize lipid bilayers through the formation of non-bilayer structures, resulting in perturbations of the membrane structure [45–47]. In particular, Das and Rand [48] have postulated that even small amounts of DG can induce structural transitions in model membranes, causing the lipids to spread apart and exposing the hydrocarbon core to the aqueous surface. It is apparent from the TEM images that major structural destabilization similarly occurs in the mixed DMPC/PDA vesicles. In natural membrane systems, such structural rearrangements induced by PLC (and DG) are proposed to play a major role in the initiation of membrane fusion [22].

Perturbation of the PDA matrix by PLD appears to follow a different pathway. On the basis of the ^1H NMR results, PA, which is the enzymatic reaction product, seems to remain trapped in the vesicle matrix, thereby changing the interfacial surface charge from zwitterionic (neutral) to anionic. Calcium ions, present in the buffer solution, are expected to have a high affinity for the resulting negatively charged interface. The disappearance of the ^{31}P signal in the NMR spectrum of DMPC/PDA vesicles mixed with PLD (Figure 9) is consistent with the decreased mobility of the Ca^{2+}-bridged PA. The observed color change of the surrounding PDA matrix thus appears to arise from changes in lipid packing induced by contraction and ionic interactions of the catalytic products remaining in the vesicles. Significant structural effects induced by interactions of ionic salts with charged lipids have been observed previously. In Langmuir films, for example, fatty acids that form a liquid-expanded monolayer have been shown to condense and solidify in the presence of divalent cations such as calcium [49,50]. Calcium ions also form strong dehydrated complexes with bilayer membranes of phosphatidylserine, inducing bilayer crystallization [51]. In addition, previous studies have demonstrated that the double layer capacitance of phosphatidylcholine monolayers decreases upon PLD hydrolysis, due to the transition of the liquid-expanded phosphatidylcholine into the liquid-condensed PA monolayer complexed with calcium [52]. The contraction of the DMPC lipids embedded in the PDA vesicles is also expected to produce the cracks and pits observed in the TEM image (Figure 10).

The colorimetric response of PDA vesicles is driven, ultimately, by perturbation of the original lipid packing, which is induced by the activity of phospholipases in the system described here. The mechanisms by which the lipid packing is altered, however, appear to be different for each of the enzymes investigated. Although products of PLA_2 hydrolysis are removed from the vesicle, PLC destabilizes the vesicle through formation of DG, and the products of PLD become condensed upon interaction with calcium ions. Controlling the PDA colorimetric response by altering lipid packing offers a new way to exploit these systems for biosensor applications.

Significance

Self-assembled conjugated polymers based on polydiacetylene (PDA) are shown to be optically responsive to the enzymatic activity of phospholipases — a ubiquitous class of enzymes that catalyze the hydrolysis of phospholipid molecules embedded in cell membranes. The PDA vesicle matrix surrounds the natural enzyme substrate, and undergoes a visible color reaction upon substrate hydrolysis. Nuclear magnetic resonance spectroscopy confirms the occurrence of interfacial catalysis by phospholipase and reveals the fate of the cleavage products. The colorimetric response of PDA vesicles is driven, ultimately, by perturbation of the original lipid packing, induced by the activity of phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD). These enzymes act specifically at water–lipid interfaces (interfacial enzymes) and are involved in diverse biochemical processes such as fat digestion, lipid metabolism, and regulation of signal transduction. The products of these enzymes have also been implicated in the development of disease conditions such as rheumatoid arthritis and pancreatitis. The color change (i.e. destabilization) of the PDA vesicles induced by these enzymes occurs by different pathways, depending on the specific enzyme. Although the PLA_2 hydrolysis products are removed from the vesicle, PLC destabilizes the vesicles through formation of diacylglycerol, and the products of PLD become condensed upon interaction with calcium ions.

Each of the three pathways leads, however, to the formation of the red polymer–vesicle suspension. This simple, one-step colorimetric detection scheme offers a new approach towards rapidly screening new enzyme inhibitor compounds simply by observation of vesicle suspensions which remain in the blue phase. In addition, the PDA–vesicles can also be used for the detection of toxins that exhibit enzyme-like activities. More generally, the results demonstrate how the material properties of conjugated polymers can be exploited to solve problems of biological interest.

Materials and methods

Reagents

PLA_2 (bee venom) was purchased from Sigma. PLC (*Bacillus cereus*) and PLD (*Streptomyces*) were purchased from Calbiochem. Solutions of 10,12-tricosadiynoic acid (TRCDA; Farchan Laboratories, Gainesville, Florida) were filtered through an 0.2 μM PTFE membrane before use to remove suspended polymer. DMPC was purchased from Sigma and used as received. All solvents were of reagent grade. De-ionized water

was purified through a Millipore uF filtration train, yielding a final resistivity of 18.2 Mohm/cm.

Liposome preparation

Stock solutions of TRCDA and DMPC were prepared in organic solvent, typically chloroform or methylene chloride. Appropriate ratios were mixed and the solvent evaporated. De-ionized water was added to the dried lipid mixture (final concentration, 1 mM total lipid) and probe sonication was performed (Sonics & Materials, Inc., Danbury Connecticut) for 10 min at 50% power using a 3 mm microprobe tip. The sample was heated to 80–90°C during sonication in order to ensure that the lipids are above the main phase-transition temperature. After sonication, the sample appears transparent or slightly opaque due to scattering from the vesicle particles. The sample was filtered hot through an 0.8 μM cellulose acetate membrane, and cooled at 4°C overnight. The sample was brought to room temperature and polymerized using 254 nm light (0.8 J/cm^2), yielding a dark blue solution of the polymerized vesicles.

Optical assay

Phospholipases (5 μl of 1.4 mg/ml dissolved in 10 mM Tris buffer, 150 mM NaCl, 5 mM $CaCl_2$, pH 8.9) were added to 50 μl of DMPC/PDA vesicles (0.1 mM final total lipid concentration, diluted in 50 mM Tris buffer, pH 7.0). Control experiments were carried out using only the buffer blanks (i.e. with no enzyme). The experiment was carried out in a standard 96-well plate using a Molecular Devices UV Max kinetic microplate reader. The absorption of the vesicle solution was monitored as a function of time at wavelengths of 620 nm and 490 nm. The decrease in absorption at 620 nm and the increase in absorption at 490 nm were used to calculate the colorimetric response [11]. The colorimetric response is defined as the relative change in the percentage of blue phase PDA after the addition of enzyme. The initial percent blue, PB_o, is defined as:

$$PB_o = A_{blue} / [A_{blue} + A_{red}] \times 100\%$$

where A is the absorbance at the wavelength of either the blue (~620 nm) or red form (~490 nm). (Note: 'blue' and 'red' refer to the visual appearance of the material, not its actual absorbance). The colorimetric response characterizes the percent conversion to the red phase at a given time:

$$CR = PB_o - PB_f / PB_o \times 100\%$$

where PB_f is the final percent blue after the enzymatic reaction. Alternatively, the reaction mixture was proportionately scaled up to allow measurement of the visible absorption spectra in a 1 ml cuvette. Spectra were recorded using a Hewlett Packard Spectrophotometer Model 9153 C.

NMR spectroscopy

NMR experiments were carried out at 27°C on a Bruker DMX500 spectrometer using $^1H/^{13}C$ /^{31}P QNP probe for ^{31}P and ^{13}C detection, and TXI probe for ^1H acquisition. The assay conditions for the NMR experiments were identical to those of the optical absorption assay, except that a 10 mM sodium borate buffer was used instead of Tris buffer. Lipid concentrations were 1 mM in 90% H_2O/10% D_2O. 200 free induction decays (FIDs) were accumulated in the ^1H experiments, whereas 10000 FIDs were acquired in the ^{13}C and ^{31}P spectra. The ^1H spectra were obtained using application of a one-dimensional NOESY pulse-sequence with water presaturation and 200 ms mixing delay. Bloch-decay pulse-sequence combined with ^1H WALTZ-16 decoupling sequence during acquisition has been applied for acquiring the ^{13}C data, whereas a Bloch decay sequence has been used in the ^{31}P experiments. TMS in water has been the external reference for ^1H and ^{13}C, and 0.1 M H_3PO_4 has been used as a reference for ^{31}P.

Transmission electron microscopy

TEM imaging of DMPC/PDA vesicles was obtained using a JEOL-100CX electron microscope under an 80 kV accelerating voltage. Formvar-coated copper grids (200 Mesh) were covered using a one-drop method: 5 μl of vesicle or enzyme–vesicle solution is dropped on the film and allowed to incubate for 3 min. The drop is removed by capillarity using filter paper. The grid is then incubated with the negative stain solution (phosphotungstic acid) for 2 min and dried, prior to imaging.

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