Formation of Oligopeptide-Based Polymeric Membranes at Interfaces between Aqueous Phases and Thermotropic Liquid Crystals

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We report a method for the synthesis of oligopeptide-based polymeric membranes at interfaces between aqueous phases and immiscible liquid crystals. The polymeric membrane was generated by contacting an aqueous solution of the oligopeptide SNKTRIDEANQRATK{Nle}L with a thermotropic liquid crystal, 4-cyano-4′-pentylbiphenyl, doped with adipoyl chloride (cross-linking reagent). We report characterization of the membrane by scanning electron microscopy and measurements of the permeability of the membrane to phospholipids. The permeability of the polymeric membrane to phospholipids was evaluated by monitoring the orientational response of the liquid crystal upon contact with an aqueous dispersion of vesicles of L-α-dilauroyl phosphatidylcholine. We measured incubation of the membrane in an aqueous solution of trypsin to cause an increase in the permeability of the polymeric membrane to the phospholipids. In contrast, polymeric membranes incubated in aqueous solutions of α-chymotrypsin and bovine serum albumin did not exhibit enhanced permeability, as revealed by the orientational response of the liquid crystals. These results suggest new materials and methods for reporting the presence of enzymes.

Introduction

The development of new materials enables novel approaches to the study of complex biological phenomena.[1] One such example, the preparation of peptide-modified interfaces, has received a great deal of attention due to the potential utility of these interfaces for monitoring enzymatic activities,[2−6] controlling cellular behaviors,[7,8] and manipulating peptide−protein interactions.[9] A number of strategies have been reported for incorporation of peptides into various interfaces, including interfaces of glass substrates,[4] polymers,[10] and metals.[11,12] In this paper, we report a strategy for incorporation of oligopeptides into interfaces of thermotropic liquid crystals. We also report that selective cleavage of oligopeptide substrates incorporated into these interfaces by enzymes can provide new strategies for reporting enzymatic activity.

The approach reported in this paper builds on past studies that have shown that liquid crystals can serve as effective amplifiers and transducers of chemical and biochemical events at interfaces.[13−17] For example, phospholipids assembled at aqueous−liquid crystal interfaces have been shown to couple strongly to the ordering of the liquid crystal, and binding and enzymatic activities of proteins at these interfaces have also been shown to lead to ordering transitions in the liquid crystal.[15,18−21] The activities of the proteins could be easily visualized between crossed polarizers because the long-range orientational order in the liquid crystal couples to the organization of the proteins and phospholipids at the interface.[18] The study reported herein sought to establish a

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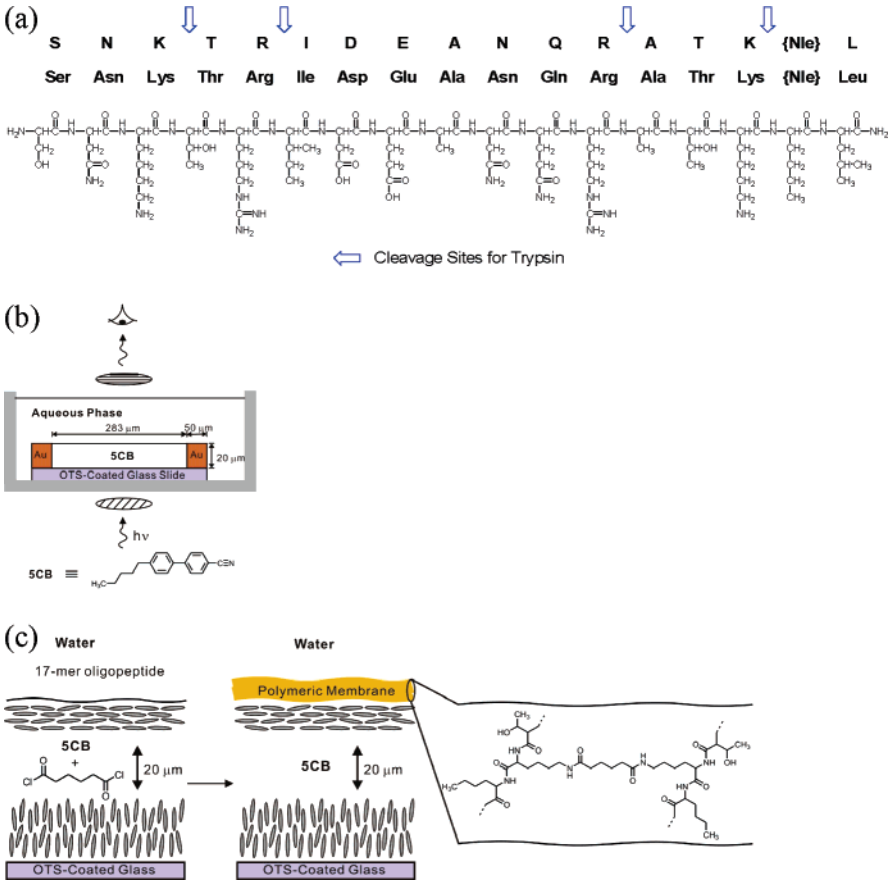

Figure 1. (a) Structure of the 17-amino acid oligopeptide used in this study, (b) schematic illustration of the experimental system, and (c) illustration of membrane formed at interface between liquid crystal and aqueous phase through the cross-linking reaction of the 17-mer oligopeptide with adipoyl chloride.

general methodology for incorporating oligopeptides at interfaces of liquid crystals with the goal of using the liquid crystal to visually amplify enzymatic activities occurring at these interfaces. The method of preparation involves the addition of an oligopeptide to an aqueous phase and a cross-linking agent to a water-immiscible liquid crystal. The oligopeptide and the cross-linker react to generate a polymeric membrane at the interface between the two phases.

For our initial studies, we used a 17-amino acid oligopeptide that contains residues 187−203 of SNAP-25, a SNARE protein consisting of 206 residues (Figure 1a).[22] We used this oligopeptide because it is a substrate of botulinum neurotoxin type A (BoNT/A) metalloprotease[23,24] and our long-term goal is to exploit the materials described in this paper to report the presence of BoNT/A. In the study reported in this paper, however, we exploit the fact that this oligopeptide is also a substrate for the enzyme trypsin. We report characterization of the structure of the oligopeptide-based membrane by scanning electron microscopy (SEM), the orientational ordering of the liquid crystal in contact with the membrane, and test the permeability of the membrane to phospholipids by monitoring orientational transitions in the liquid crystals upon contact with dispersions of lipid vesicles. We sought to determine if the presence of the oligopeptide-based membrane at the aqueous−liquid crystal interface would retard the transfer of lipids into contact with the liquid crystal. We also sought to determine if the transport properties of the oligopeptide-based polymeric membrane could be manipulated by contacting the membrane with an enzyme (trypsin) that cleaves the oligopeptide substrate incorporated into the membrane.

Experimental Section

Materials. The 17-amino acid oligopeptide was synthesized at the Biotechnology Center at the University of Wisconsin−Madison using a Fmoc protocol with an Applied Biosystems Synergy 432A instrument (see Supporting Information). Deionization of a distilled water source was performed using a Milli-Q system (Millipore, Bedford, MA) to give water with a resistivity of 18.2 MΩ cm. The liquid crystal 4-cyano-4′-pentylbiphenyl (5CB) was obtained from EM Industries (Hawthorne, NY). Adipoyl chloride, octyltrichlorosilane (OTS), trypsin, α-chymotrypsin, and lysine were purchased from Aldrich (Milwaukee, WI). Bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and L-α-dilauroyl phosphatidylcholine (L-DLPC) was purchased from Avanti Polar Lipids (Alabaster, AL). The glass microscope slides and eight-well chamber slides were purchased from Fisher Scientific (Pittsburgh, PA). Gold electron microscopy grids (20 μm thickness, 50 μm bar width, and 283 μm hole width) were purchased from Electron Microscopy Sciences (Hatfield, PA).

Preparation of Optical Cells. Glass microscope slides were cleaned and functionalized with OTS according to the previously reported procedures.[14] The OTS-coated glass slides were cut into pieces (ca. 5 mm × 5 mm) and the pieces were fixed on the bottom

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of each well of an eight-well chamber slide with epoxy. Gold electron microscopy grids were then placed onto the OTS-coated glass slides. To prepare 5CB doped with adipoyl chloride, adipoyl chloride was added to 5CB and homogeneously mixed using a vortex mixer. The 5CB doped with adipoyl chloride (1.3 wt %) was dispensed onto the grids supported on the OTS-coated glass slides, and the excess liquid crystal was removed by using a capillary tube. To prepare oligopeptide-based polymeric membrane at the liquid crystal interface, an aqueous oligopeptide solution (0.20 mM, pH 11.0) was quickly introduced into the well with a syringe. The oligopeptide was reacted with the interface of the adipoyl chloride-doped liquid crystal for 2 h. After reaction, the aqueous solution was exchanged five times with water before any other aqueous solution was introduced into the well. Aqueous solutions were exchanged such that the meniscus did not fall below the liquid-crystal interface to prevent the displacement of the liquid crystal from the grid.

Characterization. The optical images of liquid crystals were monitored between crossed polarizers of an optical microscope (BX60, Olympus, Tokyo, Japan). The optical images were captured using a digital camera (Olympus C-4000 Zoom) with consistent settings of the microscope (50% of maximum intensity, 10% open aperture, 4× magnification) and camera (*f*-stop of 2.8 and shutter speed of 1/320 s). The orientations of liquid crystals were quantitatively characterized from the average retardance measured by using an optical microscope (BX41, Olympus, Tokyo, Japan) equipped with a retardance imaging system (LC-PolScope, CRI, Woburn, MA). Scanning electron microscope (SEM) images were obtained using a LEO 1550 VP field-emission SEM at 1.00 kV.

Preparation of Aqueous Dispersions of Phospholipids. L-DLPC in chloroform (50 mg/mL) was evaporated from a glass vial under a stream of nitrogen to afford a lipid film on the vial wall. The lipids were then dried under vacuum for 2 h. The dried lipids were resuspended in water (pH 8.0), resulting in a turbid dispersion (0.10 mM). Probe sonication of the dispersion (3× at 15 W for 5 min) resulted in a clear solution, which was filtered using a 0.22 μm filter (Millipore) before use.

Results and Discussion

Figure 1b shows the experimental system used to prepare the oligopeptide-based membrane at the interface between the liquid crystal and aqueous phase. To polymerize the oligopeptide at the aqueous−liquid crystal interface, adipoyl chloride was added to the liquid crystal 4-cyano-4′-pentylbiphenyl (5CB, Figure 1b) and homogeneously mixed by using a vortex mixer. Past studies have established that the reaction of primary amine groups of oligopeptides with adipoyl chloride can lead to the formation of amide interpeptide linkages (Figure 1c).[25] We confirmed reaction of lysine and the 17-amino acid oligopeptide with adipoyl chloride by ^{1}H NMR (see Figures S-3 and S-4 in the Supporting Information). In the studies reported in this paper, we used 1.3 wt% adipoyl chloride in the 5CB. We measured the clearing temperature (T_{ni}) of the 5CB to decrease from 34.4 ± 0.2 °C to 32.7 ± 0.2 °C upon addition of 1.3 wt % adipoyl chloride to the 5CB. The 5CB doped with adipoyl chloride (1.3 wt %) was deposited into the pores of gold electron microscopy grids supported on octyltrichlorosilane (OTS) coated glass slides. An aqueous solution of oligopeptide (0.20 mM, adjusted to pH 11.0 with NaOH) was contacted with the 5CB to initiate cross-linking.

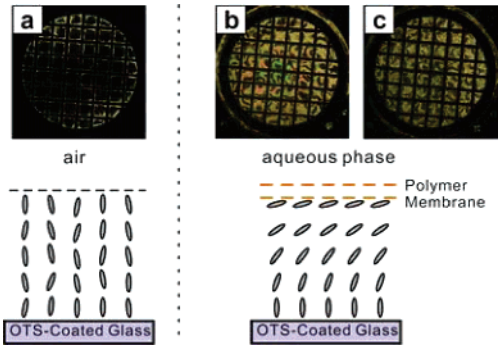

Figure 2. Polarized light micrographs of 5CB (crossed polars) mixed with adipoyl chloride (1.3 wt %) (a) before contact with aqueous solution and after (b) 0 min and (c) 2 h of contact with an aqueous 17-mer oligopeptide solution (0.2 mM, pH 11.0). The schematic illustrations below the optical images indicate the orientation of the liquid crystal within the film.

Figure 2 shows the optical appearance of the liquid crystal during polymerization. All optical images reported in this paper were obtained (in transmission mode) between crossed polarizers (Figure 1b). Before immersion under the aqueous solution containing oligopeptide, the 5CB doped with adipoyl chloride appeared dark between crossed polarizers (Figure 2a). Since it is known that OTS-coated glass slides cause perpendicular (homeotropic) anchoring of 5CB,[14,26] the dark appearance in Figure 2a is consistent with a perpendicular orientation of the liquid crystal at the interface with air.[19] When 5CB assumes a perpendicular orientation at both interfaces, the liquid crystal cannot rotate the plane of polarization of the incident light, thus leading to a dark appearance between crossed polars.[21] Immediately after contact with the aqueous oligopeptide solution, the optical appearance of 5CB became bright (Figure 2b) and remained bright during 2 h of contact with the solution (Figure 2c). This result indicates a tilted or parallel orientation of the liquid crystal at the aqueous−5CB interface. In this situation, the tilt of the 5CB (as measured from normal) must vary continuously across the thickness of the sample (see the illustrative representation below parts (b) and (c) of Figure 2).[19,21,27] We sought to quantify the tilt of 5CB at the aqueous−liquid crystal interface in the presence of the oligopeptide. The tilt angles were determined by measurement of the retardance of the liquid crystals using a microscope equipped with a retardance imaging system (LC-PolScope, CRI, Woburn, MA). The tilt angle of 5CB at the aqueous−liquid crystal interface was calculated from the retardance and thickness of the liquid crystal (20 μm).[19,26,28] In contrast to surfactants that we have observed to cause a range of tilt angles (measured as a function of increasing surfactant concentration),[19,21,29,30] we measured the introduc-

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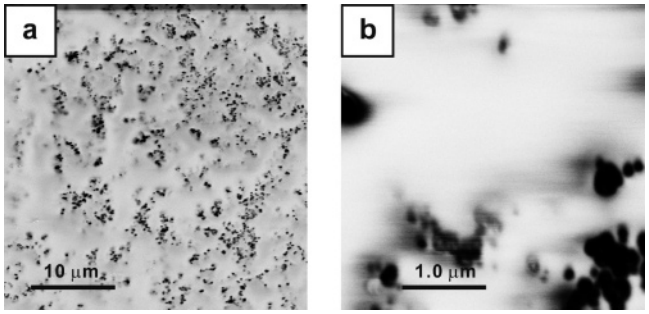

Figure 3. SEM images of the polymer membrane obtained at two different magnification levels (see scale bars).

tion of the oligopeptide to not perturb the orientation of the liquid crystal from that measured with water in the absence of oligopeptide (parallel to the interface). We also observed the interference colors of the 5CB to not change during the polymerization of the oligopeptide in the presence of adipoyl chloride: the unknown effect of the adipoyl chloride on the birefringence of 5CB, however, prevented us from quantitative determination of the tilt of the liquid crystal in the presence of adipoyl chloride. We note here that the dark brushes seen in Figures 2b and 2c indicate that the *azimuthal* orientation of the liquid crystal varies across each compartment of the electron microscopy grid.[18−21,29,30] We performed two additional control experiments in which we (i) added adipoyl chloride to the 5CB in the absence of oligopeptide in the aqueous phase (no polymer membrane formed) and (ii) added peptide to the aqueous phase in the absence of adipoyl chloride in the 5CB (no polymer membrane formed). These results support the conclusion that reaction of the adipoyl chloride and oligopeptide lead to the formation of the peptide-based membrane at the interface between the 5CB and aqueous phase.

We observed the membrane formed at the interface between the 5CB and aqueous phase to be soft and gel-like. We measured the mass fraction of water within the hydrated membranes (see Supporting Information) to be 0.63 ± 0.13 (averaged over five samples), consistent with the qualitative observation that the membranes are gel-like. We also characterized the structure of the oligopeptide-based membranes by scanning electron microscopy (SEM). Figure 3 shows SEM images of the polymer membrane. These samples were prepared by rinsing the top side of the polymer membrane with deionized water five times after the aqueous phase of oligopeptide was removed. The electron microscopy grid supporting the polymer membrane was then immersed into hexanes to remove the liquid crystal. After the membrane was dried at room temperature (overnight), SEM images were obtained using a LEO 1550 VP field-emission SEM at 1.00 kV. The SEM images reveal the membrane to have a porous structure (Figure 3). The apparent diameters of the pores were measured to range from 50 to 200 nm, although we caution that each of the steps in the sample preparation described above likely perturb the sizes of the pores in these membranes. By obtaining SEM images at 45° from the surface normal (see Supporting Information), we determined the thickness of the dried membrane to be approximately 1.5 μm.

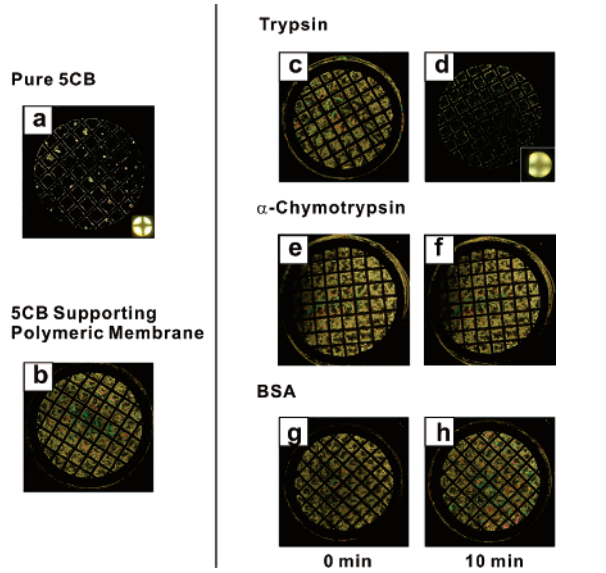

Figure 4. Polarized light micrographs (crossed polars) of (a) 5CB and (b) 5CB supporting a cross-linked oligopeptide membrane, after 30 min of contact with an aqueous dispersion of vesicles of L-DLPC (0.10 mM, pH 8.0). Polarized light micrographs of 5CB supporting a cross-linked oligopeptide membrane after incubation with indicated protein solutions (21 μM, pH 8.0) for 2 h at room temperature, and subsequent contact with an aqueous dispersion of vesicles of L-DLPC (0.10 mM, pH 8.0) for (c), (e), and (g) 0 min and (d), (f), and (h) 10 min at room temperature.

We next investigated the permeability of the oligopeptide-based membranes to transport phospholipids from the aqueous solution to the interface of the liquid crystal. The approach was based on the results of our past studies which revealed that contact of an aqueous dispersion of L-α-dilauroyl phosphatidylcholine (L-DLPC) with 5CB led to the spontaneous transfer of the lipids onto the aqueous−5CB interface and homeotropic alignment of 5CB at the lipid-laden interface.[15,20] We prepared dispersions of the phospholipid vesicles (diameters 36 ± 2 nm) by a previously reported method (see Experimental Section).[20,31,32] Next, we contacted the dispersion of vesicles with the oligopeptide-based membrane and monitored the orientational behavior of 5CB between crossed polarizers. We hypothesized that the polymer membrane fabricated at the aqueous−5CB interface would prevent or slow the transfer of phospholipids onto the interface of the 5CB. In the absence of the oligopeptide-based membrane, the optical appearance of the 5CB became black within 10 min of contact with the dispersion of lipids, indicating homeotropic alignment of 5CB (Figure 4a). The homeotropic alignment was confirmed by a conoscopic image (black cross in the inset of Figure 4a). In contrast, in the presence of an oligopeptide-based membrane, the optical texture of 5CB remained bright following 30 min of contact with the vesicles of L-DLPC (Figure 4b). After about 1 h of contact with the dispersion of the phospholipid vesicles, the 5CB supporting the oligopeptide-based membrane started to show the initial signs of small black domains, indicating the onset of transfer of lipids onto the aqueous−5CB interface. These results lead us to conclude that the formation of the oligopeptide-based membrane does slow transport of the lipid to the interface.

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We hypothesized that the barrier properties of the oligopeptide-based polymeric membrane could be manipulated by contacting the membrane with an enzyme that cleaves the oligopeptide substrate incorporated into the membrane. We sought to test this proposition by using trypsin. Trypsin is an enzyme that is known to cleave peptide bonds after (on the C-terminal side of) lysine (K) and arginine (R) if the next residue is not proline (P).[33,34] The 17-mer oligopeptide used in this research contains four cleavage sites for trypsin (Figure 1a). We incubated an aqueous trypsin solution (~21 μM, pH 8.0 for 2 h) in contact with an oligopeptide-based membrane prepared at the interface of the 5CB, removed the trypsin solution, and then added an aqueous solution containing a dispersion of vesicles of L-DLPC (0.10 mM, pH 8.0). The initial bright optical appearance of 5CB between crossed polars indicates that the orientation of 5CB is nearly planar at the aqueous−5CB interface prior to contact with the lipid (Figure 4c). In contrast, after 10 min of contact with the aqueous dispersion of vesicles of L-DLPC (0.10 mM, pH 8.0), the optical texture of 5CB became black (Figure 4d), indicating a transition to a homeotropic orientation of the 5CB. The homeotropic anchoring was also confirmed by the conoscopic image (black cross in the inset of Figure 4d). This result suggests that the polymeric membrane was processed by trypsin in a manner that facilitated the transport of phospholipid onto the interface of the liquid crystal. Control experiments with chymotrypsin and BSA (see Supporting Information for details) did not show the orientational transition to homeotropic alignment upon contact with the L-DLPC dispersion (Figure 4e−h). We used SEM to characterize changes in structure of the membrane as a result of treatment with trypsin. First, we used SEM to determine that the thickness of the dried membrane decreased from 1.5 to 0.8 μm upon incubation with trypsin. Second, we used SEM to characterize the numbers and sizes of pores apparent in images of the dried membranes. We observed that some regions of the membrane treated with trypsin appeared to possess greater numbers of pores and pores of larger size (see 2000× image shown in Figure S-5), although heterogeneity in the porous structure of the membrane prevented us from reaching an unambiguous conclusion.

The results above, when combined, suggest that the orientational transition of 5CB observed with the sample treated with trypsin (Figures 4c and 4d) arises from the selective cleavage of the polymer membrane by trypsin. More generally, the result suggests that oligopeptide-based polymeric membrane formed at interfaces of liquid crystals can be used to differentiate enzymes when an appropriate peptide substrate is employed. The approach also permits the preparation of polymeric membranes that incorporate more than one peptide. In contrast to fluorescence imaging methods, the method described here does not require labeling of substrates. This method also does not require complex instrumentation to report the enzyme activity. We briefly tested for changes in the permeability of the oligopeptide-based membrane as a function of concentration of trypsin (2 nM to 21 μM). After 30 min of contact with L-DLPC (0.10 mM, pH 8.0), the sample incubated in 2 nM trypsin solution showed small homeotropic domains. The samples incubated in trypsin solutions with higher concentrations (20 nM, 0.2 μM, and 21 μM) exhibited obvious transitions to homeotropic anchoring in the presence of lipid, clearly indicating the enzymatic degradation of the polymeric membrane by trypsin. These results suggest that it is possible to detect trypsin in the low nanomolar range with this unoptimized system. In future studies, we will investigate the use of oligopeptide-based membranes to report the activities of metalloproteases including BoNT/A.

Conclusions

In summary, we have demonstrated a novel method to prepare oligopeptide-based polymeric membranes at interfaces between thermotropic liquid crystals and aqueous phases. The permeability of the oligopeptide-based membrane to phospholipids was determined by monitoring the orientational response of the liquid crystal to the transport of phospholipids across the membrane. The results show that the oligopeptide-based membrane generated at the aqueous−liquid crystal interface retards the transport of phospholipids to the liquid crystal interface. The transport properties of the oligopeptide-based membrane could be selectively manipulated by incubating the membrane in enzymes that were specific for the oligopeptide substrates incorporated in the membrane.

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Supporting Information Available: Synthesis of oligopeptide, cleavage test, reaction of lysine and 17-amino acid oligopeptide with adipoyl chloride, measurement of water content, SEM images of the oligopeptide-based membrane, preparation of phospholipid vesicles, and control experiments using chymotrypsin and BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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