



## Hydrogels for in situ encapsulation of biomimetic membrane arrays

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# Hydrogels for *in situ* encapsulation of biomimetic membrane arrays

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Hydrogels are hydrophilic, porous polymer networks that can absorb up to thousands of times their own weight in water. They have many potential applications, one of which is the encapsulation of freestanding black lipid membranes (BLMs) for novel separation technologies or biosensor applications. We investigated gels for *in situ* encapsulation of multiple BLMs formed across apertures in a hydrophobic ethylene tetrafluoroethylene (ETFE) support. The encapsulation gels consisted of networks of poly(ethylene glycol)-dimethacrylate or poly(ethylene glycol)-diacrylate polymerized using either a chemical initiator or a photoinitiator. The hydrogels were studied with regards to volumetric stability, porosity, and water permeability. All hydrogels had pore sizes around 7 nm with volumetric changes >2% upon crosslinking. Photoinitiated hydrogels had a lower hydraulic water permeability compared to chemically initiated hydrogels; however, for all hydrogels the permeability was several-fold higher than the water permeability of conventional reverse osmosis (RO) membranes. Lifetimes of freestanding BLM arrays in gel precursor solutions were short compared to arrays formed in buffer. However, polymerizing (crosslinking) the gel stabilized the membranes and resulted in BLM arrays that remained intact for days. This is a substantial improvement over lifetimes for freestanding BLM arrays. Optical images of the membranes and single channel activity of incorporated gramicidin ion channels showed that the lipid membranes retained their integrity and functionality after encapsulation with hydrogel. Our results show that hydrogel encapsulation is a potential means to provide stability for biomimetic devices based on functional proteins reconstituted in biomimetic membrane arrays. Copyright © 2010 John Wiley & Sons, Ltd.

**Keywords:** biomimetic membrane arrays, encapsulation, hydrogel, stabilization, voltage clamp

## INTRODUCTION

Transmembrane proteins have many functions in biological systems, and act for example as channels, receptors, or transporters. Recently the notion of biomimetic membranes as technological devices incorporating essential features of biological membranes has attracted considerable interest, for recent reviews see Ref. [1–4]. Working biomimetic membranes require that the protein must be in its native (functional) conformation. This implies that for transmembrane proteins, the protein must be embedded in a matrix compatible with protein structure and function [5–7]. Black lipid membranes (BLMs) formed across single or multi-aperture hydrophobic scaffolds closely resemble the natural environment of membrane spanning proteins. However, freestanding BLMs have notoriously low longevity, which limits many applications, for comprehensive texts on BLMs see Refs. [8,9]. A biomimetic membrane working as a sensor/separation device should be stable and typically be able to withstand osmotic/hydrostatic pressures. This is not compatible with freestanding BLMs.

In order to improve BLM longevity and stability in multi-aperture arrays, we investigated the encapsulation of BLMs in an *in situ* polymerized hydrogel. Hydrogels are hydrophilic polymer networks that can absorb up to thousands of times their dry weight in water [10–12]. Hydrogels have been shown to allow transport of water, electrolytes, and possibly proteins depending on the pore size of the hydrogel network [10,13]. Recently encapsulation of single freestanding biomimetic membranes

has been proven to be a successful approach [13–23]. In this work we characterize several hydrogels and show successful hydrogel encapsulation of multiple BLMs formed across an aperture array.

In order for biological membranes to maintain their liquid crystalline state they generally require ambient temperatures between 10 and 40 °C [24]. Although the use of block-copolymers could extend the temperature range for the biomimetic protein matrix *per se*, the proteins themselves will generally denature

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with increased temperatures<sup>[25]</sup>. Thus, a required property for a hydrogel for encapsulating biomimetic membranes is that it is liquid and crosslinkable *in situ* at room temperature. Poly(ethylene glycol)-di(meth)acrylate (PEG-D(M)A) based hydrogels fulfill this requirement. Amongst other requirements is that the crosslinking process should not result in large gel volume changes as this would destabilize the biomimetic membrane. In addition the porosity of the encapsulating gel should be able to provide sufficient stability while preserving good water and solute permeabilities.

We have studied the encapsulation potential of PEG-1000-DMA and PEG-400-DA hydrogels. In particular, we characterized chemically induced and UV-induced polymerized hydrogels with respect to volumetric stability, water permeability, and porosity. We obtained stable hydrogel encapsulated lipid bilayers with lifetimes of several days in an aperture array with 64 individual BLMs as evidenced by monitoring conductance and capacitance using standard voltage-clamp measurements<sup>[26,27]</sup>. Functionality of the encapsulated BLMs was demonstrated by recording single channel activity of incorporated gramicidin ion channels.

## EXPERIMENTAL

### Materials

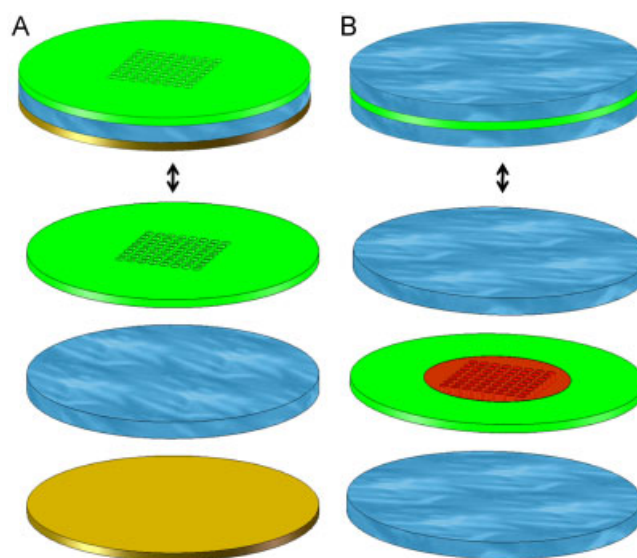
Poly(ethylene glycol)-1000-dimethacrylate (PEG-1000-DMA) and poly(ethylene glycol)-400-diacrylate (PEG-400-DA) were from Polysciences (Warrington, PA, USA). 2-Hydroxy-2-methyl-1-phenyl-propan-1-one (Darocur<sup>®</sup> 1173) was from Ciba Specialty Chemicals (Basel, Switzerland). KCl, HCl, 1,4-Piperazinediethanesulfonic acid (PIPES), ethylenediamine-tetraacetic acid (EDTA), protease inhibitor cocktail (P2714), sodium hydroxide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), *n*-decane, ethanol, and ammonium persulfate (APS) were from Sigma Aldrich Denmark (Brøndby, Denmark). Multi-aperture arrays were fabricated from Tefzel ethylene tetrafluoroethylene (ETFE) LZ200 (DuPont, Detroit, USA). Sheets of cellulose membrane on a propylene support (DSS-RC70PP) were from Alfa Laval (Nakskov, Denmark). For the retention experiments 1 kDa beads were from Merck-Schuchardt (Hohenbrunn, Germany) and 3–35 kDa beads from Fluka (Buchs, Switzerland).

For the encapsulated bilayer experiments, Viton A fluoroelastomer used for the production of rubber chamber-sealing O-rings was from DuPont Fluoropolymers (Detroit, USA). Uncoated 35 and 50 mm glass-bottom culture dishes were from MatTek (Ashland, MA, USA). 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-acrylate (DPhPE-A) were from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and gramicidin was from Sigma Aldrich Denmark (Brøndby, Denmark).

### Methods

#### Preparation of hydrogels

Hydrogels were studied in two experimental configurations (see Fig. 1). For the porosity and water flux characterization, the configuration consisted of a hydrogel sandwiched between a DSS-RC70PP membrane and a partition with an 8 × 8 aperture array fabricated from ETFE as previously described<sup>[28]</sup> (see Fig. 1a). For the BLM encapsulation experiments BLM arrays were formed across the ETFE partitions and subsequently gel encapsulated (see Fig. 1b). The two configurations can be seen as prototypical



**Figure 1.** Schematic representations of the hydrogel sandwich and of the hydrogel-encapsulated lipid membrane (closed and exploded views). (a): The sandwich consists of an ETFE partition  $\varnothing$  21 mm with an 8 × 8 aperture (aperture diameter 300  $\mu$ m) array (green), a crosslinked hydrogel (blue) and a regenerated cellulose membrane ( $\varnothing$  21 mm) on polypropylene support (brown) positioned with the cellulose side toward the gel and the polypropylene side away from the gel. (b): For BLM encapsulation a BLM (semi-transparent red circular area) is formed across the aperture array in the ETFE partition (green disk) and encapsulated<sup>Q1</sup> by hydrogel on both sides (blue disks). This figure is available in colour online at [wileyonlinelibrary.com/journal/pat](http://wileyonlinelibrary.com/journal/pat).

for future applications: a sandwich supported membrane for separation applications where hydrostatic pressures are applied (Fig. 1a.), a free-standing encapsulated membrane for sensor applications, where no (or low) pressures are applied.

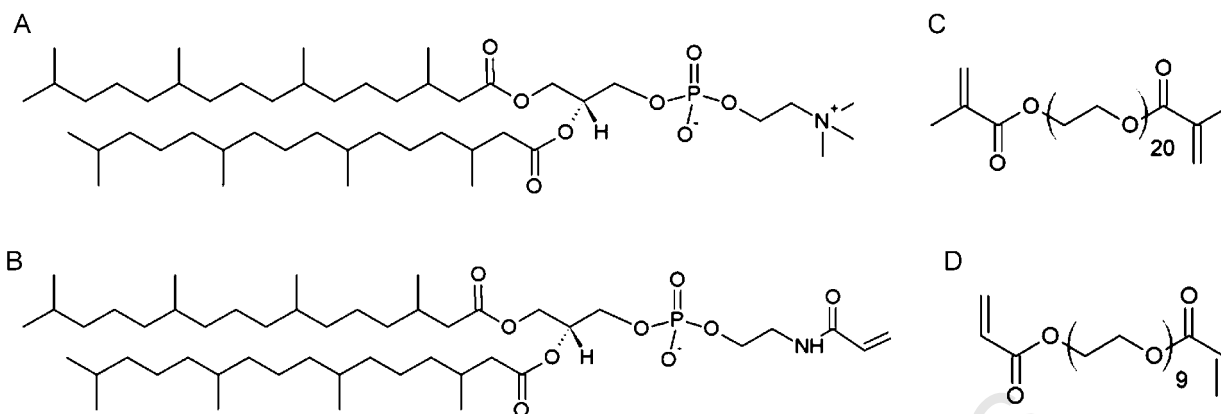
For all hydrogels, concentrations of PEG and initiator were selected so that the hydrogels crosslinked within 10 min of initiation. Table 1 and Fig. 2 present the hydrogels used in this study.

All hydrogel precursor solutions (HPSSs) were prepared in a buffer containing 25 mM PIPES, 2 mM EDTA, 0.2 M KCl and protease inhibitor cocktail in MilliQ water, adjusted to pH 6.5 using HCl.

For photoinitiation, two aqueous HPSSs were prepared: a PEG-1000-DMA solution (1000P) containing 65 mM poly(ethylene glycol)-1000-dimethacrylate monomers and 10 mM Darocur1173 photoinitiator and a PEG-400-DA solution (400P) containing

**Table 1.** The four hydrogel precursor solutions (HPSSs) investigated. For details see the Materials and Methods section

Sample	Polymer	Initiator
400P	PEG-400-DA 100 mM	Darocur 5 mM
400C		TEMED 15 mM, APS 15 mM
1000P	PEG-1000-DMA 65 mM	Darocur 5 mM
1000C		TEMED 10 mM, APS 10 mM



**Figure 2.** Chemical structures of lipids and polymers used. (a): 1,2-di-(3,7,11,15-tetramethylhexadecanoyl)-sn-glycero-3-phosphocholine (DPhPC). (b): 2-di-(3,7,11,15-tetra-methylhexadecanoyl)-sn-glycero-3-phosphoethanolamine acrylate (DPhPE-A). (c): Poly(ethylene glycol)-1000-dimethacrylate (PEG-1000-DMA). (d): Poly(ethylene glycol)-400-diacrylate (PEG-400-DA).

100 mM poly(ethylene glycol)-400-diacrylate monomers and 10 mM Darocur1173 photoinitiator.

Similarly, for chemical initiation two aqueous HPSs were prepared: a PEG-1000-DMA solution (1000C) with 65 mM PEG-1000-DMA monomers and 10 mM TEMED and a PEG-400-DA solution (400C) containing 100 mM PEG-400-DA monomers and 15 mM TEMED. Ammonium persulfate (same concentration as TEMED in 400 and 1000 C, respectively) was added to initiate polymerization.

In this paper we will refer to the individual HPSs using the nomenclature presented in Table 1. Crosslinking is indicated by the prefix X. Thus, for example, X1000P refers to PEG-1000-DMA crosslinked using UV photo-activation.

#### Volumetric stability measurements

A glass capillary (diameter 1 mm, length 15 cm) was filled with 50  $\mu$ l of HPS. Free-radical polymerization of 1000P and 400P was initiated by 5 min irradiation of the HPS by an EA-140 UV lamp with  $I = 800 \mu\text{W cm}^{-2}$  at 15 cm,  $\lambda = 365 \text{ nm}$  (Spectroline, Westbury, NY, USA) at a lamp distance of 1 cm. Gelation of 1000C and 400C was initiated when APS was added to and vortexed with the HPS prior injection into the capillary. The relative change in volume,  $\Delta V$ , was defined as

$$\Delta V = \frac{V_2 - V_1}{V_1} 100\% = \frac{L_2 - L_1}{L_1} 100\% \quad (1)$$

where  $V_1$  and  $L_1$  are the initial volume and length and  $V_2$  and  $L_2$  are the final volume and length of the hydrogel cylinder in the capillary.  $L_1$  and  $L_2$  were measured as the distances between the bottoms of the two menisci using graph paper.  $\Delta V$  was determined for the crosslinking reaction at room temperature ( $\Delta V_X$ ) and for cooling of the crosslinked sample from room temperature to 4  $^\circ\text{C}$  ( $\Delta V_{X,\text{Cool}}$ ) for each sample.

#### Preparation of a hydrogel sandwiched between ETFE and DSS-RC70PP membrane supports for retention and water permeability measurements

The sandwich assembly consists of hydrogel sandwiched between a perforated ETFE structure and a sheet of DSS-RC70PP membrane (see Fig. 1a).

Microstructuring of the ETFE support structure was performed as described in Ref. [28]. Briefly, a carbon dioxide laser was used to

fabricate 21 mm diameter partitions of ETFE LZ200 film (50.8  $\mu\text{m}$  thick) with a centered  $8 \times 8$  rectangular aperture array. The apertures had a center-to-center distance of 400  $\mu\text{m}$  and a diameter of  $300 \pm 5 \mu\text{m}$ . A disk with a diameter of 21 mm was cut out of a sheet of a DSS-RC70PP membrane and sonicated 10 min in 60/40 ethanol/water followed by 10 min sonication in water. The prewetted DSS-RC70PP membrane was placed with the cellulose side positioned upwards facing the hydrogel solution.

For the photoinitiated hydrogel sandwiches, 1 ml of HPS was distributed evenly on the DSS-RC70PP membrane. An ETFE partition (as described above) was placed on top of the liquid HPS. Then the sandwich was UV irradiated using the EA-140 UV lamp described above at a distance of 1 cm for 10 min. Chemically initiated hydrogel sandwiches were prepared in a similar manner. After 10 min the crosslinked sandwiches were packed in water-soaked cotton wool and stored at 4  $^\circ\text{C}$ .

An empty sandwich (no hydrogel) made up of an ETFE partition with an  $8 \times 8$  aperture array on top of a sheet of DSS-RC70PP (cellulose side toward ETFE) served as control.

#### Retention measurements

A feed solution containing a mixture of 1, 3, 5, 8, 12, and 35 kDa poly(ethylene glycol) (PEG) beads each at a concentration of 0.5 mg/ml in MilliQ water was passed through the hydrogel sandwich using a homemade water flux measurement setup.

Size exclusion chromatography was performed using a 717PLUS Autosampler configured with a 600E controller and a 410 Refractive Index Detector from Waters Corporation (Waters, Milford, MA, USA). Measurements were done using Waters Ultrahydrogel 250 6  $\mu\text{m}$  7.8  $\times$  300 mm<sup>2</sup> GPC column with MilliQ water as eluent at a flow rate of 0.6 ml min<sup>-1</sup> and a sample injection volume of 40  $\mu$ l. Three consecutive permeates for each hydrogel sandwich sample were collected and results reported using the third permeate chromatogram as it was identical to the second permeate chromatogram indicating that a steady-state was reached.

#### Water permeability measurements

The hydrogel sandwich was placed in a homemade flow unit cell. The time  $t$  needed to collect a given volume  $V$  of permeate

(approximately 1 ml) was measured at 1, 1.5, and 2 bars of the feed pressure  $P$ . The water flux  $J_w$  was calculated as  $J_w = \frac{V}{tV_w}$ , where  $V_w$  is the partial molar volume of water ( $1.8 \times 10^{-5} \text{ m}^3 \cdot \text{mol}^{-1}$ ). The hydraulic permeability  $L_p$  [ $\text{m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$ ] and the diffusional permeability  $P_w$  [ $\text{m}/\text{s}$ ] of the membrane were calculated from Equation (2), where  $R$  is the gas constant ( $8.3145 \text{ m}^3 \cdot \text{Pa} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ) and  $T$  is the temperature.

$$L_p = \frac{J_w V_w}{\Delta P}, \quad P_w = \frac{J_w R T}{\Delta P} = \frac{L_p R T}{V_w} \quad (2)$$

#### Formation of hydrogel-encapsulated BLMs across multi-aperture ETFE partitions

2 ml DPhPC or 1 ml DPhPE-A in chloroform ( $10 \text{ mg ml}^{-1}$ ) was placed under nitrogen flow to evaporate the solvent and the dry lipid films were rehydrated in  $400 \mu\text{l}$  and  $193 \mu\text{l}$  *n*-decane respectively to a final concentration of 59 mM. The lipid solutions were prepared 1 day prior to use and stored at  $-20^\circ\text{C}$ .

Briefly, the experimental chamber for bilayer formation consisted of an open top compartment and an open bottom compartment (35 and 50 mm culture dishes, respectively). The glass cover slip of the 35 mm glass-bottom dish was replaced with an ETFE partition with an  $8 \times 8$  aperture array by first removing the cover slip by adding 0.5 ml *n*-heptane to the dishes for 10 min, and then gluing the ETFE LZ200 partition array on the dish using silicone-based glue (Dow Corning, Midland, MI, USA). A Viton ring was placed between the two Petri dishes to create two independently accessible compartments and a reusable aluminium holder was used to clamp the upper dish/viton ring/lower dish sandwich together.

The bottom and top compartments were filled with 4.5 ml of a HPS. The experimental chamber was placed in a Faraday cage and Ag/AgCl electrodes were placed in each compartment.  $2 \mu\text{l}$  of the lipid solution was deposited over the apertures using a Pasteur pipette to form BLMs, simultaneously monitoring the current between the electrodes. To facilitate bilayer thinning, an air bubble was created at the tip of the Pasteur pipette and swept gently across the entire ETFE partition array.

When all apertures were filled with lipid membranes, an electrical seal was obtained and the capacitance ( $C$ ) and conductance ( $G$ ) of the formed membrane array could be measured by applying triangular ( $10 \text{ mV}_{pp}$ ) and rectangular ( $10 \text{ mV}_{pp}$ ) voltage clamp waveforms, respectively. Membrane capacitance values ( $C = I(dU/dt)^{-1}$ ) was determined by measuring the peak-to-peak amplitude of the square-shaped response signal, while the membrane conductance ( $G = I/U$ ) was determined from the post-transient steady-state amplitudes. Thinning of the array membranes was evidenced electrically as increase in  $C$  and optically with a light microscope with the appearance of Plateau-Gibbs borders. As the measurements recorded  $C$  and  $G$  from the entire array, rupture of even a single membrane in the array was evident as a complete loss of the electrical seal ( $G \approx 0 \Omega$ ). For details on chamber assembly and multi-array BLM monitoring and characterization see Ref. [26,27,29].

After BLM formation, encapsulation in X1000P was performed by UV irradiation of the 1000P solution with a 100 W EXFO Omnicure1000 UV-lamp (EXFO Photonic Solutions Inc, Canada). Irradiation was performed at 2% of the lamp intensity at a lamp distance of 3 cm from the ETFE partition, and with a total UV irradiation time of 15 min divided into intervals of 5 min UV

irradiation followed by 5 min intervals without UV irradiation. The experimental chamber was covered in order to minimize evaporation.

Incorporation of the channel forming peptide gramicidin into lipid membrane arrays was carried out by drying 120 nM gramicidin in ethanol under a nitrogen flow and re-dissolving to a final concentration of 3 nM in the bilayer forming solution. The gramicidin-containing multiple BLMs were encapsulated in X1000P as described above with buffer adjusted to 1 M KCl as electrolyte. For single channel recordings, a 60 mV DC potential was applied across the membranes and current traces were acquired at 1 kHz using a patch-clamp amplifier (A-M Systems, Sequim, WA, USA) and filtered through a low pass Bessel filter (Frequency Devices, IL, USA) with a 10 Hz cutoff.

## RESULTS AND DISCUSSION

We first examined material properties of the selected hydrogels relevant for the use of hydrogels as an encapsulation material for biomimetic membranes. Then we proceeded by demonstrating that crosslinked hydrogels can be used for *in situ* encapsulation of biomimetic membranes formed across multi-aperture arrays.

### Hydrogel material properties

#### Volumetric stability

Amongst the requirements for an encapsulating hydrogel is that it should not undergo large volume changes upon crosslinking as the expanding gel would exert a pressure on the lipid membrane and rupture it. We observed that the HPSs containing PEG-400-DA or PEG-1000-DMA only shrank slightly upon crosslinking ( $\Delta V_x < 1.1\%$ ) (see Table 2). Bringing the crosslinked gels from room temperature to  $4^\circ\text{C}$  resulted in further shrinkage ( $\Delta V_x < 2.0\%$ ), which exceeded the shrinkage due to crosslinking *per se*, but was still very small. The low degree of shrinkage upon gelation and temperature change is an attractive property as it makes it feasible to encapsulate biomimetic membranes and store them at  $4^\circ\text{C}$ , thus minimizing evaporation and degradation.

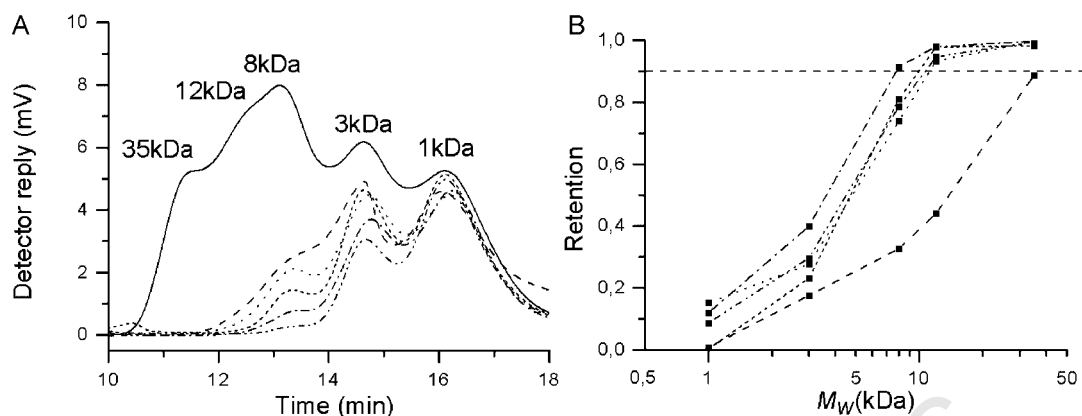
#### Polymer mesh size

The hydrogel pore sizes were characterized by passing an aqueous feed solution containing a mixture of PEG beads of various sizes at 1 bar through each assembled hydrogel sandwich (see Fig. 1a).

The permeate analysis is shown in Fig. 3. Figure 3a shows the permeate profiles for the feed, for the hydrogel sandwiches

**Table 2.** Volumetric stability of HPSs upon crosslinking. Shrinkage of each HPS upon crosslinking is expressed in percent of the volume of the HPS and upon cooling in percent of the volume of the gel. All values are given as mean  $\pm$  sd ( $n = 3$ )

Hydrogel	$\Delta V_x$ (%)	$\Delta V_{x,\text{Cool}}$ (%)
X1000P	$-0.5 \pm 0.4$	$-1.0 \pm 0.4$
X1000C	$-0.6 \pm 0.2$	$-1.3 \pm 0.4$
X400P	$-0.8 \pm 0.4$	$-0.5 \pm 0.4$
X400C	$-0.4 \pm 0.1$	$-1.4 \pm 0.6$



**Figure 3.** Hydrogel retention measurements. (a): Size-exclusion chromatograms of the feed (black line), the permeates through DSS-RC70PP(- -), X1000C (· · ·), X1000P (- · - · -), X400P (· - · - ·), and X400C (· · · · ·) hydrogel sandwiches. The bead size corresponding to each peak is indicated. (b): Retention curves for the data in (a) showing the retention for each PEG molecular weight. The cut-off level is defined as a 90% rejection level and indicated by the dashed line.

analyzed, and for the PEG bead solution when permeated through DSS-RC70PP alone. Larger beads are able to pass through the DSS-RC70PP membrane than through the hydrogel sandwich, which indicates that the bead retention  $R$  observed in the hydrogel sandwiches is caused by the hydrogels. Figure 3b shows  $R$  for the hydrogel sandwiches as a function of molecular weight:

$$R = \frac{c_f - c_p}{c_f} \quad (3)$$

where  $c_f$  is the detected peak height of the feed and  $c_p$  is the detected peak height of the permeate for each PEG bead molecular weight. The cut-off size, defined as the bead molecular weight for which 90% of the beads are retained by the sandwich<sup>[30]</sup>, was around 10 kDa for all gels and about three times lower than for the DSS-RC70PP membrane (>30 kDa). A 10 kDa bead size corresponds to a bead hydrodynamic diameter of about 7 nm<sup>[31]</sup>. This is similar to the mesh-size of PEG-1000-DMA reported previously<sup>[13]</sup>. X1000P had a marginally smaller cut-off diameter than the other gels. Thus both crosslinked PEG-400-DA and PEG-1000-DMA have nanoscale mesh structures suitable for biomimetic membrane support.

#### Water permeability

In order to ascertain whether the nanoscale mesh hydrogels would impede solute and solvent flux across the membrane, we performed water flux measurements on the hydrogel sandwiches and the results are summarized in Table 3. Fluxes were compared to the flux through DSS-RC70PP membrane alone and flux through an 'empty' sandwich consisting of a perforated ETFE disk on top of a disk of DSS-RC70PP membrane. The DSS-RC70PP membrane had a mean  $L_p$  value of  $1.65 \times 10^{-10} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$ , not significantly different from the mean value of  $1.52 \times 10^{-10} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$  measured when the perforated ETFE partition was put on top of the cellulose. The presence of crosslinked hydrogel reduced  $L_p$  for all hydrogels investigated (see Table 3). The reduction was larger for photoinitiated gels than for chemically initiated gels with no correlation between hydrogel PEG chain length/mesh size and flux reduction. The photoinitiated gels contain the Darocur 1173 initiator which is hydrophobic (c.f. CIBA

Darocur 1173 datasheet), and the reduction in water permeability may be due to photoinitiated gels having residual amounts of initiator left in contrast to the chemically initiated gels tested. The highest gel  $L_p$  was found for X400C ( $1.06 \times 10^{-10} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$ ), i.e. reduced to about 60% of the  $L_p$  of the empty sandwich whereas the lowest  $L_p = 3.4 \times 10^{-11} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$  was found for X400P. A typical reverse osmosis (RO) membrane flux is about  $12 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$  operated at 600 kPa (brackish water feed)<sup>[32]</sup> corresponding to  $L_p = 5.6 \times 10^{-12} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$ . Thus all hydrogels tested have water permeabilities at least five-fold higher than the permeability for a typical RO membrane. The hydrogels do therefore not constitute the limiting factor in biomimetic separation devices, allowing the overall device permeability (and selectivity) properties to be determined by the biomimetic membrane *per se*.

#### Hydrogels as encapsulation materials

Having established that the PEG-DMA- and PEG-DA-based hydrogels have mesh size, water flux, and volumetric stability parameters compatible with their use as encapsulation material we now investigated *in situ* encapsulation of biomimetic lipid

**Table 3.** Water permeability data. For each sample flux was measured at 1, 1.5, and 2 bars and the water permeability  $L_p$  was obtained from a linear fit to the flux-pressure relationships with  $r > 0.99$ . The effective sample area was  $3.46 \text{ cm}^2$ . All values given as mean  $\pm$  sd ( $n = 3$ )

Sample	$L_p$ ( $10^{-12} \text{ m} \cdot \text{s}^{-1} \cdot \text{Pa}^{-1}$ )	$P_w$ ( $\text{cm} \cdot \text{s}^{-1}$ )
Cellulose	$165 \pm 18$	$2.28 \pm 0.25$
Cellulose + ETFE	$152 \pm 24$	$2.09 \pm 0.33$
X1000P	$39 \pm 7$	$0.53 \pm 0.10$
X1000C	$55 \pm 19$	$0.76 \pm 0.27$
X400P	$34 \pm 14$	$0.46 \pm 0.19$
X400C	$106 \pm 41$	$1.46 \pm 0.56$
RO*	5.6	0.08

\* Based on  $12 \text{ L m}^{-2} \text{ h}^{-1}$  at 600 kPa, see Ref.<sup>[32]</sup>.

membranes. We characterized lipid membrane lifetimes in hydrogel precursor solution and lifetimes and membrane characteristics of hydrogel-encapsulated lipid membranes. We selected photoinitiated hydrogels, as the initiator can be added directly to the HPS yielding a homogeneous distribution of initiator. Also UV irradiation yielded precise control of the time point at which the polymerization reaction began.

#### Effects of HPS on BLM formation and stability

We tested both *n*-decane containing DPhPC and DPhPE-A lipid bilayers. DPhPC is a commonly used lipid for BLMs<sup>[1]</sup>. DPhPE-A contains an acrylate group which has the potential to covalently link to the acrylate group of PEG-1000-DMA or PEG-400-DA upon crosslinking.

Free-standing DPhPC and DPhPE-A BLMs in buffer had lifetimes up to 20 hr for DPhPC and up to 19 hr for DPhPE-A (see Table 5). Adding polymer to the buffer resulted in BLMs with lifetimes up to 18 hr for DPhPC and up to 14 hr for DPhPE-A (see Table 4). When both photoinitiator and polymer were present in the HPS, stability was reduced significantly with BLM lifetimes up to 139 min.

In order to identify whether the reduced BLM stability was due to specific interactions between photoinitiator and BLM, we investigated DPhPE-A membrane formation in the presence of photoinitiator alone in buffer where we obtained lifetimes ranging from 9 to 139 min. The minimum DPhPE-A membrane lifetime was substantially larger than the minimum lifetimes (seconds) observed for solutions containing PEG-based polymers with or without initiator. From this we conclude that the initiator *per se* does not compromise BLM formation, but it affects BLM lifetime. Thus the pre-encapsulation step, where both polymer and initiator are present, generally tends to destabilize the BLMs.

#### Stability of encapsulated BLMs

A sufficient minimum membrane lifetime is more critical than a maximum membrane lifetime as the membranes need to survive long enough in the HPS to become encapsulated. We selected the 1000P HPS for encapsulating BLMs, as we observed a higher minimum lifetime (albeit still < 1 min) for membranes in 1000P than in the 400P HPS.

We then encapsulated DPhPE-A and DPhPC with 1000P using UV-initiation started as soon as the membrane array was formed. The presence of X1000P seemed to “freeze” the membrane array protecting it from rupture. Once membranes were encapsulated, they all survived more than 1 day. Although several DPhPC membrane arrays ruptured during crosslinking we were able to have a DPhPC membrane array surviving in X1000P for more than a day (see Table 5), which is 12 times longer than the maximum lifetime we obtained for DPhPC membrane arrays in 1000P.

The DPhPE-A membranes generally performed better than DPhPC membranes, with lifetimes ranging from 47 hr to 3.6 days in X1000P (compared to 1 min in 1000P). Encapsulated membrane arrays were stored at room temperature with no visible degradation. The hydrogel seemed to stabilize these membranes considerably, which suggested that the gel was covalently linking to the membranes via the headgroup acrylate moiety. Alternatively, the observed stabilizing effect could be due to DPhPE-A lipids stabilized by crosslinking the lipid headgroups with each other and not due to the hydrogel. We

**Table 4.** Lifetimes of DPhPC and DPhPE-A lipid membranes in hydrogel precursor solution components. DPhPC and DPhPE-A bilayers were formed in HPS across an ETFE partition with an 8 × 8 rectangular aperture (300 μm diameter) array. The minimum and maximum observed lifetimes of membranes with C > 2000 pF are given. For each HPS composition tested, the success rate (success = survival of the membrane for at least 5 min) for these membranes is indicated as the number of successful membranes out of the total number of membranes formed

HPS	Lipid	$t_{\min}$ (min)	$t_{\max}$ (min)	$S_{(t>5 \text{ min})}$
1000	DPhPC	0.05	189	6/32
1000	DPhPE-A	1	835	14/16
1000P	DPhPC	0.08	139	2/7
1000P	DPhPE-A	0.03	1	0/9
400	DPhPC	0.03	1135	3/7
400	DPhPE-A	0.03	28	4/25
400P	DPhPC	0.02	9	1/4
400P	DPhPE-A	0.02	32	1/6
P	DPhPE-A	9	139	3/3

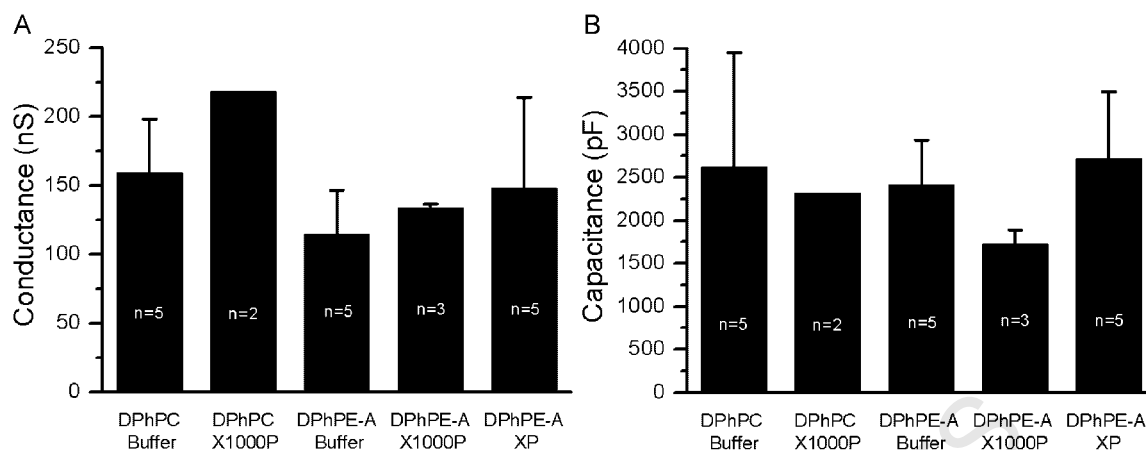
therefore analyzed DPhPE-A BLM stability in Darocur1173 solution alone exposed to the same UV dose as the membranes in hydrogel (XP solution), and obtained lifetimes ranging from 30 min to 34.8 hr. This is below the minimum lifetime obtained for DPhPE-A in X1000P, and indicates that the increased longevity of the DPhPE-A membranes is caused by the presence of encapsulating hydrogel.

In order to assess whether the hydrogel encapsulation affected BLM properties, we measured conductance *G* and capacitance *C* as described previously<sup>[27]</sup>. Membrane conductance ranged from 115 to 220 nS and membrane capacitance ranged from 1560 to 3950 pF with no significant differences between hydrogel-encapsulated membranes and freestanding membranes (see Fig. 4). This shows that encapsulation of the membranes does not change their characteristics. The capacitance values correspond to an overall specific membrane capacitance of 0.035–0.088 μF cm<sup>-2</sup> (assuming that the total membrane area was equal to the area of the aperture array, 0.045 cm<sup>2</sup>), which is

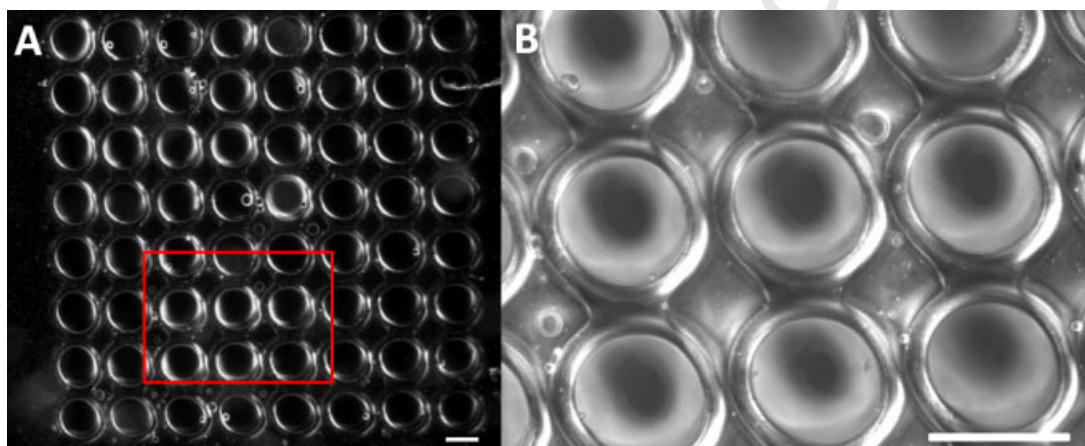
**Table 5.** Lifetimes of DPhPC and DPhPE-A lipid membranes in buffer or encapsulated in X1000P hydrogel. The minimum and maximum observed lifetimes of membranes are given. The success rate (success = survival of the membrane for at least 12 hr) for these membranes is indicated as the number of successful membranes out of the total number of membranes formed

Sample	UV time (min)	Lipid	$t_{\min}$ (hr)	$t_{\max}$ (hr)	$S_{(t>12 \text{ hr})}$
Buffer	None	DPhPC	0.02	20.8	2/6
		DPhPE-A	0.6	19.8	2/5
XP	15	DPhPE-A	0.6	37.8	2/5
		DPhPC	0.8	34.8	1/2
X1000P	15	DPhPC	0.8	34.8	1/2
		DPhPE-A	47.3	219.1	3/3





**Figure 4.** Freestanding X1000P-encapsulated BLM electrical properties. (a): conductance  $G$  (b): capacitance  $C$ . Mean  $\pm$  sd values of each membrane were determined using the methods described in Ref. [26] after 50 min for membranes with lifetime  $>60$  min or the last 10 min for membranes with lifetime  $<60$  min.



**Figure 5.** Optical microscope images of a hydrogel-encapsulated lipid membrane obtained as described in Ref. [26]. (a): The entire  $8 \times 8$  array. (b): magnified image of the apertures in the red square in (a), showing lipid membranes with clearly recognizable Plateau-Gibbs borders (light annular regions). Scale bar  $300 \mu\text{m}$ .

much lower than the specific capacitance for fully thinned solvent-containing membranes ( $0.4\text{--}0.6 \mu\text{F cm}^{-2}$ ) reported in the literature<sup>[33]</sup>. The low specific overall capacitance value measured does not preclude the existence of bilayer patches, but if they exist they only constitute 9–22% of the total aperture area and the rest of the aperture array area has solvent-containing lipid patches. This is consistent with the bright field image of the  $8 \times 8$  arrays where the Plateau-Gibbs borders<sup>[34]</sup> with large annuli are clearly visible (see Fig. 5). Of course, an optimized thinning of the BLMs would increase the effective bilayer area. On the other hand this would also tend to make membranes even

more unstable before crosslinking. Thus we are faced with a compromise between larger bilayer areas and reasonable BLM formation success rates and lifetimes.

#### Functionality of encapsulated BLMs

In order to verify that we have areas where the lipid bilayer thickness is compatible with the hydrophobic spanning segments of transmembrane proteins, we incorporated the channel-forming peptide gramicidin A into the BLMs followed by X1000P encapsulation (see Fig. 6).



**Figure 6.** Single channel gramicidin current trace from an X1000P-encapsulated 5 day old DPhPE-A membrane in buffer adjusted to 1 M KCl. A 60 mV DC potential was applied across the membrane and the signal was filtered at 10 Hz.

Current trace recordings showed single channel events with current amplitudes around 2 pA, corresponding to a single channel conductance of 33 pS, consistent with previous results for gramicidin in 1 M KCl<sup>[35]</sup>. The current trace recordings confirmed functional reconstitution of gramicidin ion channels, which strongly suggested that functional lipid bilayer areas are present in the X1000P-encapsulated and stabilized membranes.

## CONCLUSION

Hydrogels show promise as materials for encapsulating biomimetic membranes. They have volumetric stability and their nano-scale cut-off sizes and water flux properties make them promising in biomimetic devices where they can support the biomimetic membranes and yet allow vectorial flux of matter to (and through) the biomimetic membrane. We have demonstrated that multi-aperture arrays of membranes can be successfully encapsulated and that it is possible to create a robust biomimetic membrane in which proteins can be reconstituted. However, the main challenge still remains: how to improve the stability of the membranes in the HPS before crosslinking and to ensure that protein function is not compromised by maneuvers used to crosslink the hydrogels.

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## REFERENCES

[1] C. H. Nielsen, **2009**, Biomimetic membranes for sensor and separation applications. *Anal. Bioanal. Chem.* 395: 697–718.  
 [2] E. Sackmann, M. Tanaka, **2000**, Supported membranes on soft polymer cushions: fabrication, characterization and applications. *Trends Biotechnol.* 18: 58–64.  
 [3] E. Reimhult, K. Kumar, **2008**, Membrane biosensor platforms using nano- and microporous supports. *Trends Biotechnol.* 26: 82–89.  
 [4] J. Jagur-Grodzinski, **2009**, Polymeric gels and hydrogels for biomedical and pharmaceutical applications. *Polym. Adv. Technol.* 21: 27–47.  
 [5] C. H. Nielsen, Lipid-protein interactions in biomembranes. in *Handbook of Molecular Biophysics* (Eds. H. G. Bohr), Wiley, Berlin, **2009**, 329–358.  
 [6] F. N. R. Petersen, I. Laursen, H. Bohr, C. H. Nielsen, **2009**, Protein-induced bilayer perturbations: lipid ordering and hydrophobic matching. *Biochem. Biophys. Res. Commun.* 387: 760–765.  
 [7] F. N. R. Petersen, C. H. Nielsen, **2009**, Raman spectroscopy as a tool for investigating lipid protein interactions. *Spectroscopy* 24: 26–35.  
 [8] H. Ti Tien, **1974**, *Bilayer lipid membranes (BML) Theory and practice*. Marcel Dekker Inc, New York  
 [9] H. T. Tien, A. Ottova-Leitmannova, (Eds.) *Planar Lipid Bilayers (BLMs) and Their Applications*. Elsevier, Amsterdam, **2003**.  
 [10] A. S. Hoffman, **2002**, Hydrogels for biomedical applications. *Adv. Drug Delivery Rev.* 54: 3–12.

[11] N. A. Peppas, Y. Huang, M. Torres-Lugo, J. H. Ward, J. Zhang, **2000**, Physicochemical foundations and structural design of hydrogels in medicine and biology. *Annu. Rev. Biomed. Eng.* 2: 9–29.  
 [12] N. A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, **2000**, Hydrogels in pharmaceutical formulations. *Eur. J. Pharm. Biopharm.* 50: 27–46.  
 [13] T. J. Jeon, N. Malmstadt, J. J. Schmidt, **2006**, Hydrogel-encapsulated lipid membranes. *J. Am. Chem. Soc.* 128: 42–43.  
 [14] N. Malmstadt, T.-J. Jeon, J. J. Schmidt, **2008**, Long-lived planar lipid bilayer membranes anchored to an in situ polymerized hydrogel. *Adv. Mater.* 20: 84–89.  
 [15] J. W. Shim, L. Q. Gu, **2007**, Stochastic sensing on a modular chip containing a single-ion channel. *Anal. Chem.* 79: 2207–2213.  
 [16] X. F. Kang, S. Cheley, A. C. Rice-Ficht, H. Bayley, **2007**, A storable encapsulated bilayer chip containing a single protein nanopore. *J. Am. Chem. Soc.* 129: 4701–4705.  
 [17] R. F. Costello, I. R. Peterson, J. Heptinstall, N. G. Byrne, L. S. Miller, **1998**, A robust gel-bilayer channel biosensor. *Adv. Mater. Opt. Electron.* 8: 47–52.  
 [18] R. F. Costello, I. R. Peterson, J. Heptinstall, D. J. Walton, **1999**, Improved gel-protected bilayers. *Biosens. Bioelectron.* 14: 265–271.  
 [19] J. A. Beddow, I. R. Peterson, J. Heptinstall, D. J. Walton, **2004**, Reconstitution of nicotinic acetylcholine receptors into gel-protected lipid membranes. *Anal. Chem.* 76: 2261–2265.  
 [20] H. T. Tien, A. L. Ottova, **1998**, Supported planar lipid bilayers (s-BLMs) as electrochemical biosensors. *Electrochim. Acta* 43: 3587–3610.  
 [21] W. Ziegler, J. Gaburjakova, M. Gaburjakova, B. Sivak, V. Rehacek, V. Tvarozek, T. Hianik, **1998**, Agar-supported lipid bilayers - basic structures for biosensor design. Electrical and mechanical properties. *Colloids Surf., A* 140: 357–367.  
 [22] T. Baumgart, A. Offenhauser, **2003**, Polysaccharide-supported planar bilayer lipid model membranes. *Langmuir* 19: 1730–1737.  
 [23] M. Uto, M. Araki, T. Taniguchi, S. Hoshi, S. Inoue, **1994**, Stability of an agar-supported bilayer lipid membrane and its application to a chemical sensor. *Anal. Sci.* 10: 943–946.  
 [24] J. R. Hazel, **1988**, Homeoviscous adaptation in animal cell membranes. *Physiol. Regul. Membr. Fluidity* 149–188.  
 [25] Ed., T. E. Creighton, **1993**, *Proteins, Structures and Molecular Properties*, 2nd Ed., New York.  
 [26] J. S. Hansen, M. E. Perry, J. Vogel, J. Groth, T. Vissing, M. Larsen, O. Geschke, J. Emneus, H. Bohr, C. H. Nielsen, **2009**, Large scale biomimetic membrane arrays. *Anal. Bioanal. Chem.* 395: 719–727.  
 [27] M. Perry, T. Vissing, T. P. Boesen, J. S. Hansen, J. Emneus, C. H. Nielsen, **2009**, Automated sampling and data processing derived from biomimetic membranes. *Bioinspir. Biomim.* 4: 44001.  
 [28] J. Vogel, M. E. Perry, J. S. Hansen, P.-Y. Bollinger, C. H. Nielsen, O. Geschke, **2009**, Support structure for biomimetic applications. *J. Micromech. Microeng.* 19: 025026.  
 [29] J. S. Hansen, M. E. Perry, J. Vogel, T. Vissing, O. Geschke, J. Emneus, C. H. Nielsen, **2009**, Development of an automation technique for the establishment of functional lipid bilayer arrays. *J. Micromech. Microeng.* 19: 025014.  
 [30] M. Mulder, **1996**, *Basic Principles of Membrane Technology*. Kluwer Academic Publishers, Dordrecht.  
 [31] P. Szweczykowski, **2009**, Nano-porous materials from diblock copolymers and their membrane application. PhD Thesis. In Department of Chemical and Biochemical Engineering. Technical University of Denmark, Lyngby.  
 [32] L. F. Greenlee, D. F. Lawler, B. D. Freeman, B. Marrot, P. Moulin, **2009**, Reverse osmosis desalination: water sources, technology, and today's challenges. *Water Res.* 43: 2317–2348.  
 [33] R. Benz, O. Fröhlich, P. Läger, M. Montal, **1975**, Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta* 394: 323–334.  
 [34] S. H. White, **1970**, A study of lipid bilayer membrane stability using precise measurements of specific capacitance. *Biophys. J.* 10: 1127–1148.  
 [35] O. S. Andersen, **1983**, Ion movement through gramicidin A channels. Single-channel measurements at very high potentials. *Biophys. J.* 41: 119–133.

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