Research Article

Selected Paper from the 11th Pure and Applied Chemistry International Conference 2017 (PACCON 2017)

pH-Responsive Styrene Maleic Anhydride with Improved Surface Activity

Patchara Punyamoonwongsa*, Wichsuda Tangsongcharoen and Piangtawan Phoungtawee
School of Science, Mae Fah Luang University, Chiang Rai, Thailand

Brian Tighe
Biomaterials Research Group, Aston University, Birmingham, United Kingdom

* Corresponding author. E-mail: patchara@mfu.ac.th
DOI: 10.14416/j.ijast.2018.01.001
Received: 29 May 2017; Accepted: 27 July 2017; Published online: 4 January 2018

Abstract
pH-Responsive poly(styrene-alt-maleic anhydride) were synthesized and modified to improve their surface activity for transmembrane protein extraction. Different samples were synthesized by single-step solution polymerization using peroxy radical initiator, prior to esterification. The half esterified samples (ePSMA) became weaker polyelectrolytes with reduced acid dissociation constant (k_a). In the low compression region (>23 cm^2), it showed reduced surface pressures, implying weakened lateral electrostatic repulsion. It could also decrease water surface tension to 40 mN/m, beyond that of the unmodified one. The method has proved to be useful for a design of workable pH-switchable systems for a wider context of extraction technology.

Keywords: Styrene maleic anhydride, pH-Responsive, Surfactant, Protein extraction

1 Introduction

Proteins are recovered from plant tissues for different proposes. One of the most important reasons is to identify plant gene coding for genomic and physiological studies. Another reason is linked with the increased demand for protein extracts in industrial sectors, including foods and beverages, organic synthesis, biodiesel production and modern therapeutics [1]. Traditional protein extraction protocols require the use of detergent-based lysis buffer to destabilize plant lipid membranes and free the cellular proteins. The key to detergent function is relied on its amphipathic nature to adsorb and flip (or trans-membrane motion) into the membrane layer [2]. Only detergents with moderate hydrophobicity and at excess concentration can induce a slow leakage of the cytoplasmic components, such as soluble proteins, mitochondria, Golgi-apparatus, lysosomes and chloroplast [3]. Among all candidates, ionic detergents, including Sodium Dodecyl Sulfate (SDS) and Cetyl Trimethyl Ammonium Bromide (CTAB), are the most popular choice for protein extraction due to their simplicity, low-cost and high extraction capacity. However, they always lead to protein contamination and denaturation [4]. The alternative choices with less protein denaturing problem are relied on non-ionic (Triton-X) and zwitterionic (dodecyldimethyl-*N*-amineoxide, DDAO) detergents. Unfortunately, they often display low extraction capacity [4], [5]. Thus, there is a need for new non-denatured surface-active agents that can effectively extract proteins of interest at high yield, while maintaining their biological functions and native structures.

Alternating copolymer of styrene and maleic anhydride (insertion in Figure 1) or PSMA has emerged as promising surface-active agent for membrane solubilization. Upon alkaline hydrolysis (pH > 10),

Please cite this article as: P. Punyamoonwongsa, W. Tangsongcharoen, P. Phoungtawee, and B. Tighe, "pH-Responsive styrene maleic anhydride with improved surface activity," *KMUTNB Int J Appl Sci Technol*, vol. 11, no. 1, pp. 45–51, Jan.–Mar. 2018.

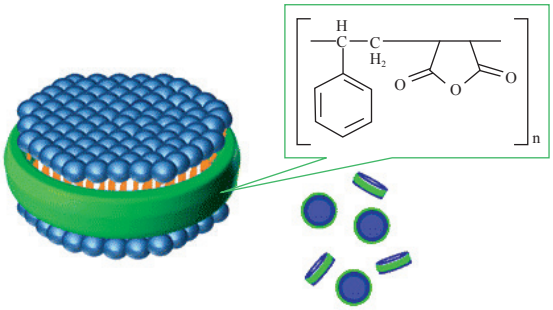

Figure 1: The reconstituted phospholipid bilayers (blue sandwich) are surrounded by the α-helical PSMA coils (circle ring), forming membrane-mimicking assemblies for protein encapsulation [6]. The insertion represents the PSMA repeating unit, containing both maleic anhydride (right) and the phenyl (left) groups.

the molecules of PSMA exist in the extended conformation due to the predominant negatively charged repulsion of the carboxylic acid groups of the anhydride (MA) moieties. At pH < 4, the molecules are protonated. Hence, the hydrophobic association among the styrene moieties dominates the electrical repulsion, resulting in a conformational transition into the amphipathic *alpha*-helical coils.

This structural arrangement allows it to hydrophobically bind, disrupt and destabilize ordinary lipid membranes. This is eventually accompanied by a slow leakage of the embedded proteins [2]. The most advantages of PSMA lysis agent is the ability to complex with native phospholipid membranes and then, form the membrane-mimicking assemblies of around 10–20 nm in diameter (Figure 1). This nanostructure enables reconstituted proteins to be encapsulated within their central lipoid zone and resolved in their full functional state.

Another interesting feature of PSMA lies on its ability to undergo chemical functionalization by many reactions [7]. The high reactivity of the MA ring in PSMA toward nucleophilic reagents allows the incorporation of various active compounds to generate a wide spectrum of new materials. Despite these, practical use of PSMA in protein extraction is still limited due to its weak surface activity in lysis buffer (pH 8–10). To overcome this drawback, we proposed a way to chemically modify PSMA structure to attain a balanced hydrophobicity/hydrophilicity groups necessarily for hydrophobically-driven membrane

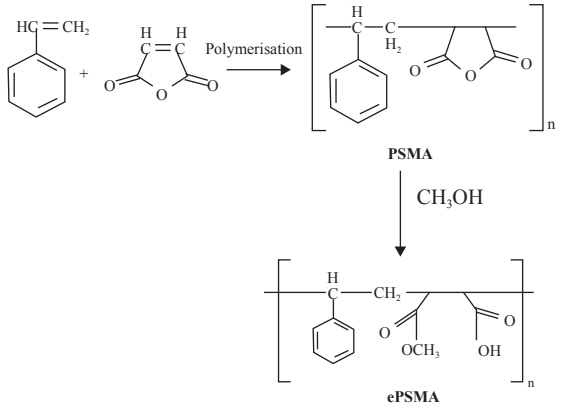

Figure 2: Synthesis and modification pathways of PSMA.

insertion. When the PSMA hydrophobic group is optimized, an acid-induced association with the lipid membrane may be unnecessary and that, the extraction of transmembrane proteins would become possible even at alkaline pH values.

This study aimed to structural design of PSMA copolymer via esterification with linear alcohol to be used as non-denatured lysis agent for transmembrane protein extraction. For this, different PSMAs were synthesized by a single-step solution polymerization using peroxy radical initiator, followed by esterification, as shown in Figure 2. The impact of esterification on material properties was evaluated through the uses of both physical and spectroscopic techniques.

2 Experimentals

2.1 *Chemicals*

Styrene (St) and Benzoyl Peroxide (BPO) were supplied from the Sigma Aldrich. Maleic Anhydride (MA) was purchased from the Fluka Analytical. Methanol (HPLC grade) and other solvents were received from the Fisher Chemical (USA). All chemicals were used without further purification.

2.2 *Synthesis of PSMA*

Equimolar mixture of St and MA was dissolved in the binary toluene/ethyl acetate solvent in the presence of BPO free-radical initiator (1.5 mol%, with respect to monomers). The reaction mixture was heated up

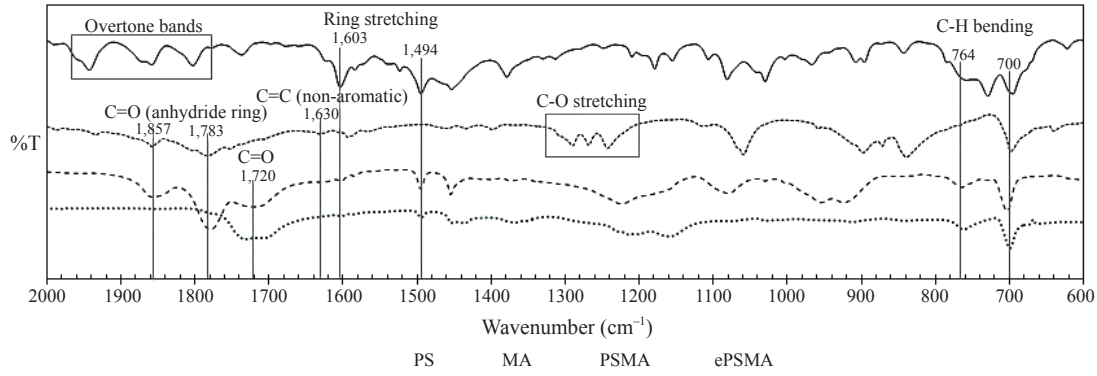

Figure 3: FT-IR Spectra of Poly(styrene) (PS), Maleic Acid (MA), PSMA and ePSMA (from top to bottom).

to around 70–80°C. After 6 h, the copolymer was precipitated in the chilled petroleum ether, filtered off and vacuum-dried at 70°C for 48 h.

2.3 *Esterification of PSMA*

The copolymer (0.10 mol, on the basis of a molar repeat unit) was dissolved in MEK solvent (50 mL) at 70–80°C, followed by the addition of the methanol (0.55 mol). After 6 h, the esterified PSMA (ePSMA) was precipitated in the chilled petroleum ether and vacuum-dried at 70°C for at least 48 h.

2.4 *Characterization*

The Fourier-Transform Infrared (FT-IR) spectroscopy (PerkinElmer Inc., USA) was used to confirm the structural conversion to the ester functional group. The dried sample was grinded with potassium bromide (KBr) powder. The IR spectra were recorded in a spectral region of 400–4000 cm^{-1} with a 32 scans and 4 cm^{-1} resolution.

The potentiometric titration method was used to determine the pK_a values of both PSMA and ePSMA. This allows the effect of esterification on material acidity and so, hydrophilicity to be evaluated. For this, the copolymers were initially hydrolyzed in aqueous alkaline solution to obtain the final concentration of 4% w/v. The pH solution was adjusted to around 13–14 by the addition of sodium hydroxide solution (1.0 M). The hydrolyzed solution was titred with hydrochloric acid (1.0 M) until the final pH value had reached to around 2. The titration curves were plotted and analyzed for the pK_a range.

The surface activity of hydrolyzed samples was investigated through the use of the Du Noüy Tensiometer and Langmuir-Blodgett Trough (Model 601M, Nima Technology Ltd., England). A stock solution (0.1% w/v) of either PSMA or ePSMA was prepared in deionized water and diluted to desired concentrations. For the tensiometric measurement, a 50 mL of the solution was placed in the sample chamber and their surface tension values were recorded and averaged based on five measurements. To form the Langmuir-Blodgett (LB) monolayer films, a 10 µL of hydrolyzed sample was injected beneath the water subphase (pH 6–7) using the Hamilton syringe. After fifteen minutes, the monolayer was continuously compressed and then expanded at a constant rate of 50 cm^2/min to obtain the surface (π) and Area (A) isotherm. Each cycle was repeated at least twice to obtain reproducible results.

3 Results and Discussions

3.1 *Structural analysis*

A successful synthesis of styrene maleic anhydride copolymer was confirmed by the FTIR technique. As shown in Figure 3, the spectrum of PSMA displayed the IR absorption bands between 1450–1500 cm^{-1} and 650–780 cm^{-1}, corresponding to the C=C stretching and the C-H bending of the aromatic ring in the St residue, respectively. The copolymer also displayed a strong absorption bands around 1783 cm^{-1} and 1857 cm^{-1}, assigned to the anhydride carbonyl (C=O stretching) in the MA residue. The characteristic IR absorption bands at 1200–1300 cm^{-1}, attributed to

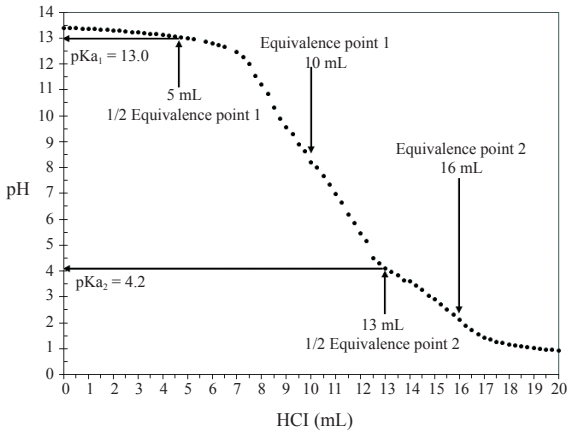

Figure 4: Potentiometric titration curve of hydrolyzed PSMA solution (4% w/v).

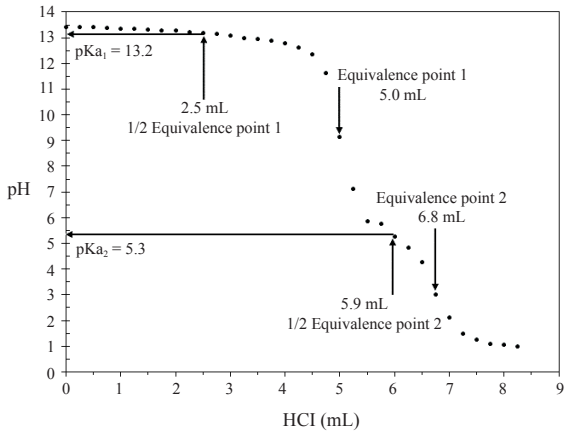

Figure 5: Potentiometric titration curve of hydrolyzed ePSMA solution (4% w/v).

the cyclic C-O stretching in MA, were also detected in PSMA sample. This suggests the presence of an unopened anhydride ring after polymerization. The coexistence of both aromatic alkene (1494 cm^{-1}, 1603 cm^{-1}) and anhydride carbonyl (1783 cm^{-1}, 1857 cm^{-1}) bands in IR spectrum of PSMA confirms the successful copolymerization between St and MA comonomers. A conversion of the cyclic anhydride to the ester carbonyl groups after PSMA esterification was convinced by the absence of the C=O anhydride ring (1783 cm^{-1}, 1857 cm^{-1}) and the broadening of the peaks at around 1720 cm^{-1}.

3.2 *Potentiometric titration*

The pK_a values of the hydrolyzed PSMA and ePSMA samples were determined from the titration curves as seen in Figures 4 and 5. To approximate the pK_a values, the equivalent points needs to be marked on the titration plot. Then, the pH at the half-equivalent point volume was identified as the pK_a value. This parameter reflects the number of carboxylic acid groups and acid strength of the materials. The larger the value of pK_a, the lesser the numbers of the acid dissociation constant (k_a) and so, the weaker acid the material become.

As seen in Figure 5, the pK_a values of ePSMA are around 13.2 and 5.3, higher than that of unmodified sample shown in Figure 4 (13.0 and 4.2). This means that ePSMA requires less amount of hydrochloric acid to protonate the acid carbonyl pendent groups and that, it behaves as weaker polyelectrolyte. This is not surprising since the side chains of ePSMA were partially substituted by the methyl group and became less ionized. The results of this potentiometric study reconfirm a structural conversion on the PSMA side chains to the ester carbonyl pendent groups thus, rendering the materials more hydrophobic for a wider context of extraction technology.

3.3 *Surface pressure and area (π-A) isotherms*

The monolayer behavior of PSMA and ePSMA was observed using the Langmuir-Blodgett Trough machine. The monolayer was initially formed by spreading hydrolyzed samples onto the water subphase (pH 6–7). After the standby period of time, the thin films were compressed and the isotherms were obtained by recording the surface pressure (π) during the barrier displacement. It is important to note that PSMA is an amphipathic molecule. When spreading on water subphase, it tends to arrange itself with the phenyl groups protruded out of aqueous phase and the carboxylic acid groups immersed within the water subphase. The ability of PSMA to form insoluble monolayer at the interface normally dictates its own surface activity and is dependent on the polymer-solvent interaction. When the molecules of PSMA are mostly ionized, the strong water interactions would drive them to be dissolved in the bulk water and not, maintained at the air-water interface. However, when they are less ionized (i.e. by methyl substitution), they would preferentially migrate from the water subphase

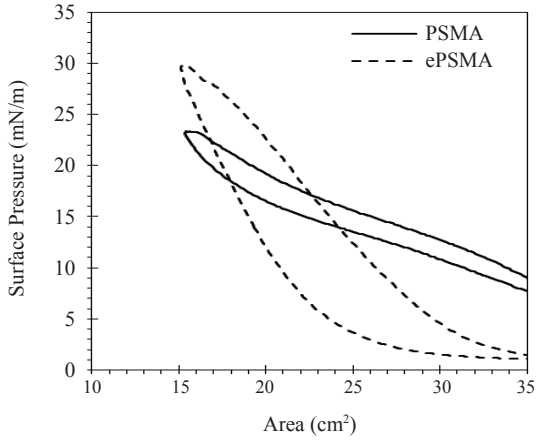

Figure 6: The surface pressure-area isotherms on deionized water subphase (pH 6–7) and at room temperature of PSMA (solid line) and ePSMA (dashed line). Monolayers were compressed at 50 cm^2/min rate.

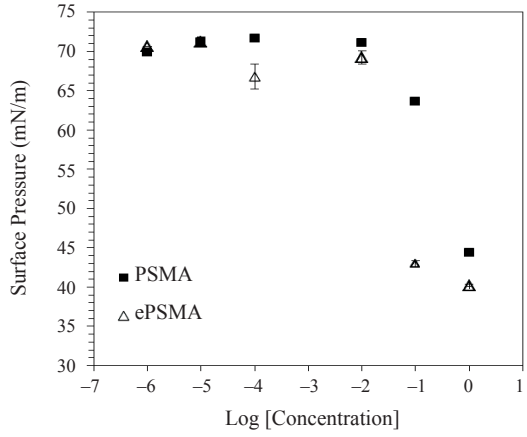

Figure 7: Comparison of the averaged-surface tension values for hydrolyzed PSMA and ePSMA samples (pH 6–7) at different concentrations.

and form an insoluble monolayer at the air-water interface.

The surface pressure-area (π-A) isotherms for PSMA and ePSMA are shown in Figure 6. For PSMA, the surface pressure at low compression region (> 23 cm^2) increases to a greater extent than ePSMA, implying the stronger lateral electrostatic cohesion in PSMA than in ePSMA membranes. The esterified PSMA is thus, seen as a weaker polyelectrolyte. This is not surprising since some of the carboxylic acid group in ePSMA had been substituted by the methyl ester group after esterification. At high compression ($\pi > 17$ mN/m), the increase of surface pressure observed in ePSMA goes far beyond that in PSMA. This is thought to be associated with the increased steric repulsion of larger methyl substituent, comparing to the hydrogen atom in PSMA. Another interesting interfacial behavior is the area relaxation of the film-forming materials. For all monolayer films, the expansion curves do not retrace the compression curves. This has been described in terms of the different between the molecular organization at compression, and the relaxation (disorganization) of molecules at decompression [8]. The area loss in relaxation due to the diffusion of the absorbed molecules to the subphase and/or the formation of multilayer structures at the air-water interface can also contribute to the hysteresis pattern of the compression-expansion isotherm. As compared to PSMA, the isotherm of ePSMA displays larger hysteresis loop. The main reason for this is ascribed to the strong hydrophobic association that promotes molecular aggregation and then, suppresses the elastic recovery of the materials.

3.4 *Surface tension*

The association and adsorption behaviour at the air-water interface of hydrolysed PSMA and ePSMA samples (10^{-6}–1% w/v) were investigated with no compression. The surface tension of the two samples at pH 6–7 against their logarithmic concentrations is shown in Figure 7. It is worth mentioning that surface activity of PSMA copolymer is generally influenced by its hypercoiling behaviour, which in turn, depends on the pH solution or a degree of ionization. It also depends on a partitioning equilibrium of the molecules between the bulk solution and at the air-water interface. In dilute solutions (< 0.01% w/v), molecules of both PSMA and ePSMA are self-aggregate in the bulk solution rather than allocated at the air-water interface. The main driving force for this is due to strong phenyl-phenyl attraction on PSMA backbone [9].

At a concentration of 0.10% w/v, the surface tension values of all samples begin to decrease, illustrating that the polymer partitioning equilibrium is shifted toward the air-water interface, and that the materials start to behave like a surface-active agent. At this concentration, a reduction of surface tension is more pronounced for ePSMA than unmodified

PSMA, as noticed by a drastic decrease in the surface tension to ≈ 43 mN/m, followed by a plateau value of ≈ 40 mN/m at a saturation concentration (C_{sat}) of 1.0% w/v. Unlike ePSMA, the surface tension profiles of unmodified PSMA shows a steady decline character with higher water surface tension. This leads to a conclusion that ePSMA possesses stronger surface activity. The surface coverage of PSMA at the air-water interface is presumably independent of the polymer chain length, but rather influenced by the polymer chain architecture. The incorporation of the methyl ester moieties on ePSMA via esterification potentially increases the short-range hydrophobic attraction and hence, promotes the local chain folding to a more surface-active form.

As mentioned earlier, the PSMA surface activity is relied on the pH solution. At pH 4, one would expect the material to possess the greatest surface activity. This is because the free carboxylate groups of the copolymer are fully protonated and the phenyl-phenyl interactions dominate over the electrostatic repulsion of the carboxylate groups. The polymer chains thereafter adopt themselves to an amphipathic helix coil conformation with the phenyl rings located on one facet and the carboxyl groups located along the opposite facet. This structural arrangement allows them to be adsorbed at the air-water interface and reduce the water surface tension to around 42 mN/m thus, acting as a surface-active agent [10]. Our results showed that PSMA adsorption at the air-water interface could be promoted without acidification. Through esterification, the copolymer (ePSMA, Figure 7) could reduce the surface tension of water (pH 6–7) to 43 mN/m, as low as that occurred in the acidic solution. A structural manipulation via esterification is thus, another key to trigger and enhance PSMA surface affinity in a biologically compatible manner.

4 Conclusions

Copolymer of Styrene and Maleic Anhydride (PSMA) was successfully synthesized by a free-radical solution polymerization, followed by esterification. The esterified product (ePSMA) showed increased pK_a values (13.2 and 5.3), suggesting that its conformational transition could become possible without acidification. A methyl substitution could render the copolymer into a weaker polyelectrolyte with improved surface activity. This was confirmed by a reduced surface tension of water (pH 6–7) to 43 mN/m, as low as that in the case of PSMA at pH 4. The structural manipulation of PSMA through esterification was indeed a key to enhance the material surface activity acquired for a design of workable pH-switchable systems for protein extraction technology.

Acknowledgments

This work was supported by a grant from the Newton Fund (A Researcher Links Travel Grant 2015). The authors would like to convey special appreciation to the academic committee of Pure and Applied Chemistry International Conference (PACCON2017) for providing the opportunity for this work to be published in this journal.

References

[1] S. Seth, D. Chakravorty, V. K. Dubey, and S. Patra, "An insight into plant lipase research-challenges encountered," *Protein Expression and Purification*, vol. 95, pp. 13–21, Mar. 2014.

[2] D. Lichtenberg, H. Ahyayauch, and F. M. Goñi, "The mechanism of detergent solubilization of lipid bilayers," *Biophysic Journal*, vol. 102, no. 2, pp. 289–299, Jul. 2013.

[3] S. Kalipatnapu and A. Chattopadhyay, "Membrane protein solubilization: Recent advances and challenges in solubilization of serotonin$_{1A}$ receptors," *International Union of Biochemistry and Molecular Biology Life*, vol. 57, no. 7, pp. 505–512, Jul. 2005.

[4] A. R. Long, C. C. O'Brien, K. Malhotra, C. T. Schwall, A. D. Albert, A. Watts, and N. N. Alder, "A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs," *BMC Biotechnology*, vol. 13, no. 41, pp. 1–10, May. 2013.

[5] J. M. Dörr, M. C. Koorengevel, M. Schäfer, A. V. Prokofyev, S. Scheidelaar, E. A. W. van der Cruijsen, T. R. Dafforn, M. Baldus, and J. A. Killian, "Detergent-free isolation, characterization, and functional reconstitution of a tetrameric K^+ channel: The power of native nanodiscs," *Proceedings of the National Academy of Sciences*, vol. 111,

pp. 18607–18612, Dec. 2014.

[6] M. Tanaka, A. Hosotani, Y. Tachibana, M. Nakano, K. Iwasaki, T. Kawakami, and T. Mukai, "Preparation and characterization of reconstituted lipid–synthetic polymer discoidal particles," *Langmuir*, vol. 31, no. 46, pp. 12791–12726, Nov. 2015.

[7] J. Jeong, B. Y. Seop, and Y. S. Lee, "Synthesis and modification of some heterocyclic compound," *Reactive and Functional Polymers*, vol. 50, pp. 257, 2002.

[8] A. P. Girard-Egrot and L. J. Blum, "Langmuir-blodgett technique for synthesis of biomimetic lipid membranes," in *Nanobiotechnology of Biomimetic Membranes*, D.K. Martin Ed. Springer US, pp. 23–74, Dec. 2007.

[9] G. Garnier, G. Garnier, M. D.-S. R. Vyhnalkova, T. G. M. van de Ven, and J.-F. Revol, "Assoication in solution and adsorption at an air-water interface of alternating copolymers of maleic anhydride and styrene," *Langmuir*, vol. 16, pp. 3757–3763, Mar. 2000.

[10] V. Saez-Martinez, P. Punyamoonwongsa, and B. J. Tighe, "Polymer-lipid interactions: Biomimetic self-assembly behaviour and surface properties of poly(styrene-alt-maleic acid) with diacylphosphatidylcholines," *Reactive and Functional Polymers*, vol. 94, pp. 9–16, Sep. 2015.