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Structural Modification of Styrene Maleic Anhydride Copolymers for Plant Bioactive Compound Extraction

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Abstract. Ability of poly(styrene-*alt*-maleic anhydride) (PSMA) to undergo a conformational transition into an amphipathic α-helix coil offers one possible mechanism by which PSMA surface activity can be switched on or off in response to the pH change. This behaviour allows it to be useful in membrane solubilization for extraction technology. Bioactive compounds are recovered from plant tissues for different reasons. One of the most important reasons is due to the increased demand in nutraceuticals market and modern therapeutics. Despite this, aqueous-based extraction of these compounds has been reported to give low extraction yield. A development of new green extraction protocol is still a challenging task for all researchers nowadays. This study demonstrated, for the first time, possible use of PSMA as a lysis agent for plant bioactive compound extraction. To enhance its membrane affinity at physiological pH, the polymer was esterified with methanol. Both PSMA and its derivative (ePSMA) were characterized in terms of their membrane binding affinity through a combined use of both surface characterization and physical techniques. Analysis of the ternary phase diagrams suggested that ePSMA could facilitate stronger hydrophobically-driven interactions with the lipid. This was convinced by the reduced critical PSMA/lipid mass ratio from 7:1 (PSMA) to 1:1 (ePSMA), as observed in the ternary phase diagrams. Last but not the least, the crude extracts of *Coffea robusta* leaves obtained from ePSMA-based extraction showed a total phenolic content of 20.32±0.75 mg/g sample, significantly higher than that from the PSMA- (14.24±1.27 mg/g sample) and aqueous-based (16.33±1.03 mg/g sample) extractions. A structural manipulation of PSMA is thus a key to tailor its membrane solubilization and so, the extraction efficacy of bioactive compounds from plant cells.

Introduction

Human body is composed of variety of macromolecules that can change their conformation and function in response to stimuli factors. Such behaviour can be reproduced by weakly charged synthetic polymers. In an aqueous solution, these polymers can form an extended structure as a result of electrostatic repulsion of the charged segments. If they also contain alkyl pendent groups, the molecules can also undergo hydrophobic association (another words, hypercoiling) and collapse into distinct hydrophobic microdomains. The ability of such polymers to hypercoil or hydrophobically associate to form compact chains offers one possible mechanism by which macromolecules can be made to change their function in response to local stimuli. One of many good example is poly(styrene-*alt*-maleic anhydride) or PSMA (Fig.1a). Above its pK_a (pH 4), hydrolyzed PSMA forms an **extended** structure due to electrostatic charged repulsion of the carboxylate functional groups. As the pH solution is lowered, the proportion of negatively charged group falls and the hydrophobic forces between the phenyl side chains becomes a predominant factor. The extended chains then collapse and eventually rearrange themselves into an **α-helix coil** (so-called hypercoil) with the **hydrophobic** phenyl groups presented along one facet and the **hydrophilic** anionic groups present along the opposite facet, as shown in Fig.1b. Such behaviour

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allows the formation of amphipathic structure analogous to native pulmonary surfactant (SP-B) found in human body [1]. Hypercoiled PSMA is usually surface active thus, leading to the possibility that PSMA surface activity could be **switched on** or **off**, in response to pH change [1, 2]. A series of pH-dependent surface activity for PSMA was given as; pH 4 > pH 6 > pH 12 [2]. Application of PSMA in lipid membrane solubilization was first suggested by Tonge and Tighe [1]. The key idea was relied on the amphipathic nature of hypercoiled PSMA that allows it to hydrophobically bind and then flip into the membrane bilayer. This would lead to a bilayer pore formation and then, a slow leakage of cytoplasmic components from the cells. The concept of PSMA-induced membrane destabilization could be very useful in development of new membrane lysis agents for water-based extraction. One of the most advantages of PSMA lysis agent is associated with its susceptibility to complex with native lipid assemblies and then form membrane-mimicking nanostructures. These particles allow poorly water-soluble compounds (i.e., transmembrane proteins and polyphenols) to be encapsulated within the core structure and then, resolved in their full functional state [1-2]. Despite this, the process of PSMA-induce membrane solubilization still requires a lowering of pH solution. This could, in turn, lead to protein denaturation, hydrolytic degradation or dimerization of active compounds. The way to solve this problem is relied on the molecular design of PSMA to achieve optimal hydrophobicity/hydrophilicity balance acquired for such transition. The incorporation of longer aliphatic side chains into PSMA may help to induce a proportionate increase in the hydrophobic forces, and limits the incursion of PSMA into water phase to greater extent. The subsequent chain folding of PSMA with those sizeable pendent groups would then be much easier to occur. As suggested by Tonge and Tighe [1], the relative efficiency of pendent groups in forming hydrophobic center has been suggested as follows; saturated aliphatic chain > unsaturated chain > aromatic groups. This concept is quite useful for a design of workable pH-triggered PSMA surface activity for both biotechnology and biomedical applications.

This study aimed to modify the PSMA structure to enhance its potential use as a membrane lysis agent for plant bioactive compound extraction. For this, the polymer was esterified with the primary linear alcohol to generate a wider range of the products with different degrees of esterification (DE). One of the most important properties of PSMA with regard to their applications is the manner in which it interacts with membrane layer. To evaluate this, the phase diagrams of PSMA/water/phospholipid were construct based on a titration method. Last but not the least, practical implementation of the materials in plant extraction was demonstrated on *Robusta* coffee leaves. This PSMA-based technology, once complimented, can lead to a development of new water-based extraction protocol with no use of surfactant, nor organic solvent.

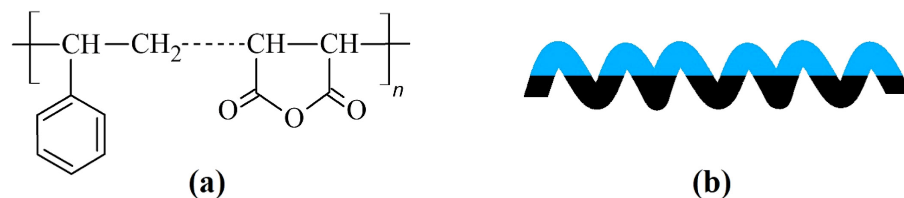

Figure 1. Chemical structure of poly(styrene-*alt*-maleic anhydride) (a), and its amphipathic coil arrangement (b), showing hydrophobic (grey) and hydrophilic (black) facets.

Experimental Methods

Materials. Poly(styrene-*alt*-maleic anhydride) (PSMA; MW 1600 g/mol, 50:50) was purchased from Polyscience (Warrington, PA). Dimyristoyl phosphorylcholine (DMPC; ≥99%) were obtained from Avanti Polar Lipid Inc. (Alabaster, Alabama, USA). Methanol (HPLC grade) and other solvents were obtained from Fisher Scientific (Loughborough, UK). Hydrolyzed sample was obtained by dissolving the polymer in NaOH (1 M) for 24 hr, before neutralizing with HCl (1 M).

Esterification of PSMA. A 0.10 mole of PSMA was dissolved in a 50 mL of methyl ethyl ketone (MEK), followed by the addition of the methanol (0.55 mol). The reaction was performed at 65°C

under continuous agitation. The degree of esterification (DE) was monitored as a function of time by pipetting a 2 mL of the reaction mixture at regular intervals. These samples (ePSMA) were precipitated with petroleum ether (4ºC), filtered and dried at 60ºC for at least 48 hr.

Structural Analysis and Ternary Phase Diagrams. The fourier-transform infrared (FT-IR) spectroscopy was used to approximate the conversion of the anhydride group. The IR spectra of the dried samples were recorded in a spectral region of 4000-600 cm^{-1} with a 32 scans and 4cm^{-1} resolution. To construct a phase diagram of DMPC and PSMA (or ePSMA) in deionized water, a 1 mL of an aqueous phospholipid solution (0.5%, 1.0%, 1.5%, 2.0% and 2.5% w/v) was titred with hydrolyzed polymer solution (6% w/v). At every 10 min intervals, a 0.1 mL aliquot of the polymer solution was slowly added to a turbid suspension of DMPC under continuous stirring. A titration was carried out until a single phase solution was obtained.

Plant Extraction. Leaves of *Coffea robusta* Pierre ex Froehner L. were collected from Mae Fah Luang University Botanical Garden in Chiang Rai, Thailand. The 3rd mature and healthy leaves from the tip were washed, chopped into small pieces and grinded in liquid nitrogen. The leaf powder (2 g) was transferred into the tube containing 10 ml of the lysis buffer. To remove any cell debris and heavy membrane fragments, the suspension was centrifuged (4ºC) at 4,000 rpm and then at 12,000 rpm. The supernatant was collected for further analysis. To prepare the lysis buffer, the 3-morpholino propanesulfonic acid (MOPS, 0.2 M), 10% glycerol, dithiothreitol (2 mM) and 1.0% (w/v) of selected surfactant (1.0% w/v, either Triton X-100, PSMA, or ePSMA) were mixed in deionized water (200 mL). The pH of all lysis buffers was adjusted to around 6.5-7.0.

Total Phenol Content. The Folin-Ciocalteu method modified from Katsube et.al [3] was used. Briefly, a 100 µL of diluted crude extract was mixed with 2 mL of Na_2CO_3 solution (2% w/v). To the solution, a 100 µL of Folin-Ciocalteu reagent (50% w/v) was added and incubated for 30 min. Color development was determined at specific absorbance (λ_{max} 750 nm). The total phenolic intensities were based on gallic acid equivalent values (mg GAE/g sample).

Total Flavonoid Content. The total flavonoid content of crude extract was determined by the aluminum chloride ($AlCl_3$) colorimetric method [4]. Briefly, the crude sample (500 µL) was mixed with a 200 µL of $AlCl_3$ solution (0.55 M) and a 100 µl of sodium nitrite solution (3 M). To this solution, a 500 µl of sodium hydroxide (2.5 M) was added. The final volume was adjusted to 2.5 mL with distilled water and incubated at room temperature for 40 min. The total flavonoid content was based on catechin equivalent value (mg/g sample) measured at the λ_{max} of 510 nm.

Results and Discussions

Structural Analysis. FT-IR technique was used to confirm a structural modification of PSMA. As shown in Fig.2, the new characteristic IR bands at around 1710-1733 cm^{-1}, attributed to the ester carbonyl group, are detected in all ePSMA samples (Fig.2). This implies a successful conversion of the cyclic anhydride to the ester group after 3, 6 and 30 hr of methylation. However, the IR bands at 1852 cm^{-1} and 1790 cm^{-1}, corresponding to the unopened cyclic anhydride (C=O stretching), remain observed in all samples. This suggests a formation of partially esterified products at the end. To monitor the changes in the degree of esterification (DE) over time, the reaction mixture was sampling at 2, 4, 6, 24, 27 and 30 hr. The value of DE was determined in terms of a conversion ratio (p) [5], which was defined as; $p\ (\%) = (1-A_t/A_0) \times 100$. The term A_t is the absorbance ratio at 1852 cm^{-1} (maleic C=O stretching) and 1602 cm^{-1} (aromatic C=C stretching), at any time given of esterification. The term A_0 is the absorbance ratio of those corresponding signals at t=0 (initial time). Results in Table 1 indicates that the absorbance ratio decreases substantially over time. This is not surprising since nucleophilic alcohol selectively reacts with the carbonyl carbon of cyclic anhydride, and not with the styrene unit. As observed, a kinetic of PSMA esterification shows a two-phase change characteristic. The first phase, occurred within the first 6 hr, is a gradual increasing of a conversion, followed by a declined rate of conversion before reaching to a plateau region. Due to an angle strain of a five-membered anhydride ring, methylation could even occur

within the first 2 hr (37% DE, 68°C). After 24 hr, a conversion of the ester reaches a plateau value of around 65%. This critical limit could hardly be raised up without the use of catalyst.

Table 1. Changes of absorbance and the ester conversion (p) upon PSMA esterification.

Absorbance	Reaction time (hr)						
	0	2	4	6	24	27	30
A1852	0.0583	0.0424	0.0335	0.0366	0.0223	0.0249	0.0263
A1602	0.0456	0.0529	0.0527	0.0636	0.0499	0.0526	0.0582
A1852/A1602 ratio	1.2790	0.8014	0.6356	0.5750	0.4475	0.4730	0.4510
A_t/A_0	1.0000	0.6266	0.4969	0.4495	0.3499	0.3698	0.3526
p (%) = $(1 - A_t/A_0) \times 100$	0	37.34	50.31	55.05	65.01	63.02	64.74

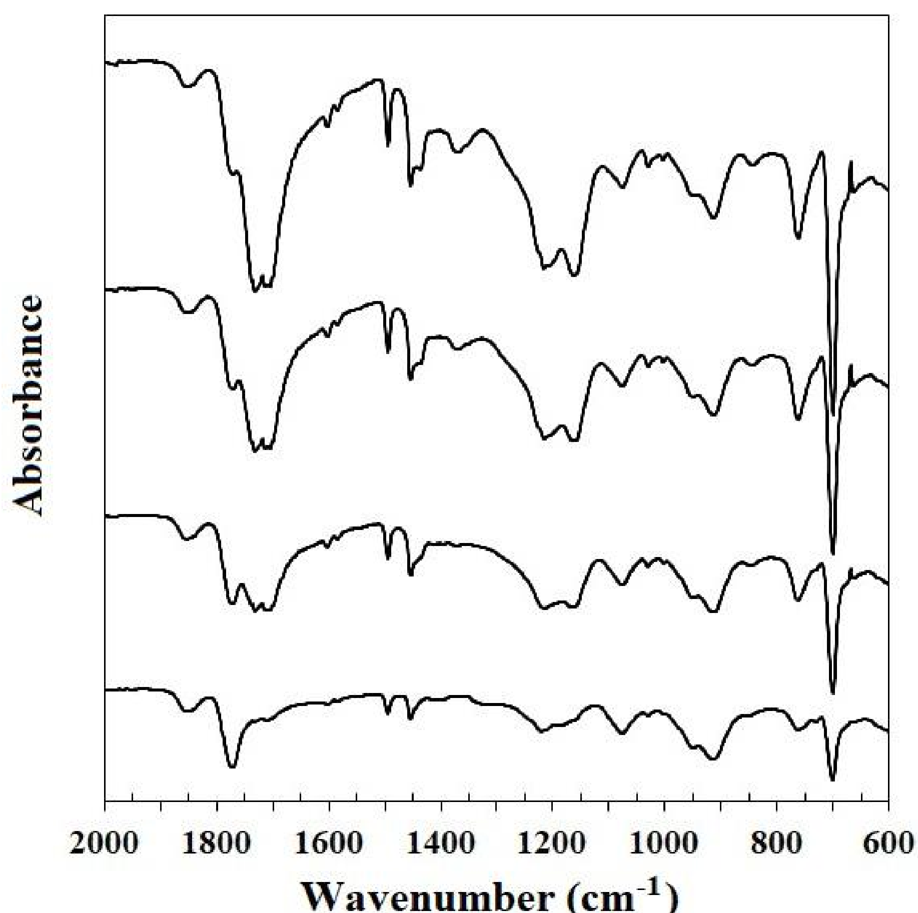

Figure 2. FT-IR Spectra of PSMA after esterification for 0, 3, 6 and 30 hr (from bottom to top).

Phase Behaviour. Fig.3 shows the high water content ternary phase diagrams of DMPC in the presence of either PSMA, or ePSMA (64.74% DE in Table 1). The single phase formulations, illustrated by a black dot on the phase diagram, represent a successful complexation between the polymer and DMPC. This was based on the fact that stable complexes with nanometric sizes (≅ 50 nm diameter) do not scatter the light and consequently, produce a homogeneously clear formation. As noticed in Fig.3, a single phase region of PSMA system is smaller than that of ePSMA system, illustrating greater ability of the latter to interrupt, destabilize and hydrophobically associate with ordinary phospholipid assemblies. The binding affinity of PSMA is normally modulated by the balance of hydrogen bonding, electrostatic and hydrophobic interactions. At neutral pH values, the carboxylic acid groups of PSMA are deprotonated thus, generating the charge repulsion with the lipid head groups. This potentially disrupts hydrophobic polymer-lipid association and reduces the extent of membrane insertion. To facilitate sufficient hydrophobically-driven interactions, an excess amount of the polymer is required under non-acidic conditions. For

PSMA-based system, the minimum PSMA/DMPC mass ratios, estimated at any single phase boundary, are found to be around 7:1. The added methyl ester group reduces this ratio to around 1:1, implying greater ability of ePSMA for membrane solubilization. The reason for this is perhaps attributed to the reduced charge repulsion by methyl ester substitution and so, the stronger hydrophobic effects in the esterified system. Under this circumstance, the insertion of the polymer within the acyl-chain packing of the lipid membrane would become more accessible, thus explaining a larger single phase region in ePSMA system shown in Fig.3a.

Phenolic and Flavonoid Contents. Plant phenolics are one group of the secondary plant metabolites, which play a major role in the protection of oxidation processes as antioxidants [6]. These compounds have received much attention as promising naturally-derived phytochemical antioxidants. Their superior biological properties include antibacterial, anti-inflammatory and antihyperglycemic. Surfactant-based protocols are still one of the most favorite techniques in bioactive compound extraction from plants. This is because of their simplicity, convenience and low cost. The choice of surfactant depends on the type of plant tissues, nature of the active compounds and the end-use applications. The key function of surfactants (so-called, lysis agent) to lyse and release the plant active compounds is relied on their amphipathic nature that allows them to bind, disrupt and flip into the cell membrane. Once the compounds are detached from the cells, they should ideally be transferred to new reservoir, resemble to their native environment. As seen in Table 2, the contents of phenolics and flavonoids in the crude extracts of *Robusta* coffee from the PSMA and ePSMA extractions are significantly higher than that from the most commonly used surfactant (Triton-X). This could be related to a formation of the membrane-like nanodiscs of the copolymers and the reconstituted lipid membrane bilayer [7, 8]. These biomimetic nano-sized assemblies can immobilize the plant phenolics within the inner cores, and render the compounds into water-soluble formulations, without affecting their functionality and native structures. Since micellar structures of Triton-X do not represent lamellar bilayers of natural membrane, the detached compounds, once deliberated, may undergo undesirable reactions and loss their functionality. Those reactions could be dimerization, oxidative degradation and structural transition.

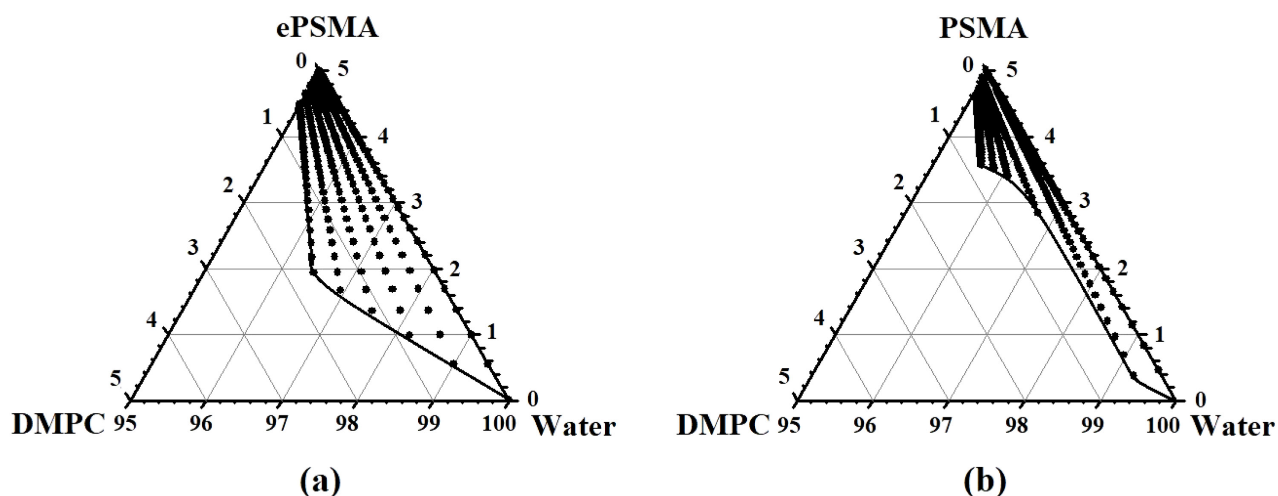

Figure 3. Ternary phase diagrams of; (a) ePSMA/DMPC/Water and (b) PSMA/DMPC/Water systems. The single phase solutions were indicated by a black dot on the phase diagram.

Table 2. Contents of total phenolics and flavonoids in the crude extracts.

Extraction media	Total phenolic content [(GAE (mg/g sample)]*	Total flavonoid content (mg/g sample)]*
Distilled water	16.33±1.03^{c}	4.39±0.63^{b}
Lysis buffer/DI water	17.91±1.15^{c}	5.02±0.54^{c}
Lysis buffer/Triton-X	10.58±0.92^{a}	2.17±0.14^{a}
Lysis buffer/PSMA	14.24±1.27^{b}	5.43±0.08^{c}
Lysis buffer/ePSMA	20.32±0.75^{d}	6.87±0.34^{d}

*Values expressed as means ± standard deviations (n = 3).
Mean followed by the same letter are not significant differences at the 0.05 level.

As mentioned in the phase behaviour study, the methyl ester substitution in PSMA had rendered the material stronger affinity for membrane destabilization. These empirical results are also consistent with those obtained by the plant extraction study. As shown in Table 2, the contents of both plant phenolics and flavonoids extracted by ePSMA are around 20.32±0.75 mg/g sample, and 6.87±0.34 mg/g sample, respectively. These amounts are significantly higher than that extracted by PSMA. Upon esterification, the active anhydride functional groups of PSMA are likely to be transformed to the hydrophobic alkoxy side chain. The incorporated ester moieties would increase the material hydrophobicity and interfacial binding capacity. For instance, a binary blend of PSMA and high-density poly(ethylene) (HDPE) is generally not miscible due to their different chemical structures. By introducing the ester group to the acid anhydride, the long alkyl side chains could facilitate hydrophobically intermolecular interactions with HDPE matrix thus, preventing undesirable aggregation in the blend films. As the degree of substitution was increased, the pK_a was shifted upward [9, 10], confirming that the derivatives had become more hydrophobic with greater capacity for binding. The engineering design of PSMA structure via esterification has thus proven to be a useful tool to improve its membrane capacity more suitable for solvent-free extraction technology.

Summary

This study demonstrated a way to structurally design of PSMA via esterification to optimize its hydrophobicity/hydrophilicity balance acquired for membrane destabilization. The half methyl ester substitution (65% DE) lowered the minimum PSMA/DMPC mass ratio from 7:1 to 1:1, suggesting greater membrane affinity and so, high potential for extraction technology. Practical implementation of the esterified PSMA (ePSMA) in *Robusta* coffee extraction showed highest contents of both plant phenolics (20.32±0.75 mg/g sample) and flavonoids (6.87±0.34 mg/g sample). A logical explanation was due to a stronger hydrophobically-driven cooperative binding between ePSMA and the plant cell membranes that led to a more successful membrane solubilization. A structural manipulation of PSMA is thus a key to tailor its membrane solubilization and so, increase the extraction efficacy of plant bioactive compounds.

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References

[1] S. Tonge, B. J. Tighe, Responsive hydrophobically associating polymers: A review of structure and properties, Adv. Drug Del. Rev. 53 (2001) 109-122.

[2] P. Punyamoonwongsa, Synthetic analogues of protein-lipid complexes, Ph.D. Thesis, Aston University, 2007.

[3] T. Katsube, H. Tabata, Y. Ohta, Y. Yamasaki, E. Anuurad, K. Shiwaku, Y. Yamane, Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-ciocalteu assay, J. Agric. Food Chem. 52 (2004) 2391-2396.

[4] W.-Y. Lin, M.J. Yang, L.T. Hung, L.C. Lin, Antioxidant properties of methanol extract of a new commercial gelatinous mushrooms (white variety of *Auricularia Fuscosuccinea*) of Taiwan, Afr. J. Biotechnol. 43 (2013) 6210-6221.

[5] R.S. Rajput, A. Singh, R.K. Singh, Diesterification and biological study of styrene maleic anhydride copolymer with alcohol and DMSO. Intel. J. Chem. Tech. Res. 3 (2011) 441-447.

[6] C. Rice-Evan, N. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends. Plant Sci. 2 (1997) 152-159.

[7] S. Paulin, Surfactant-free purification of membrane protein complexes from bacteria: application to the *Staphylococcal* penicillin-binding protein complex PBP2/PBP2a, Nanotechnol. 25 (2014) 1-7.

[8] S. Scheidelaar, M.C. Koorengevel, J.D. Pardo, J.D. Meeldijk, E. Ebeukink, J.A. Killian, Molecular model for the solubilization of membranes into nanodisks by styrene maleic acid copolymers, Biophys. J. 108 (2015) 279-290.

[9] P. Punyamoonwongsa, W. Tangsongcharoen, P. Phoungtawee, pH-Responsive styrene maleic anhydride with improved surface activity, KMUTNB: Intel. J. Appl. Sci Tech. 11 (2018) 45-51.

[10] G. Chen, Y. Zhang, X. Zhou, J. Xu, Synthesis of styrene-maleic anhydride copolymer esters and their surface enriched properties when blending with polyethylene, Appl. Surf. Sci. 253 (2006) 1107-1110.