Stoichiometric and Nonstoichiometric Polyelectrolyte Complex of Chitosan and Polyethyleneglycol-Monosuccinate: Preparation and Characterization

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ABSTRACT: Stoichiometric and nonstoichiometric polyelectrolyte complex (PEC) was prepared with polyethylene glycol-monosuccinate (PEGMS), and chitosan (CS). A series of PEGMS were synthesized by a 1 : 1 mol ratio between PEG and succinic anhydride. Then, the novel PEC was prepared by a various mole reaction of the above synthesized PEGMS and CS. The physicochemical properties of the synthesized PEC was characterized by using elemental analysis, FTIR, ^{1}H, and ^{13}C nuclear magnetic resonance, dissolution behavior, and phase transition phenomenon. Furthermore, some properties of the PEC obtained were analyzed by UV-Visible spectrometry, wide-angle X-ray diffraction, differential scanning calorimeter, scanning electron microscope, and estimated solubility, and cell viability assay, respectively. It was found that the observed FTIR, ^{1}H, and ^{13}C-NMR data was in good agreement with the chemical structure of the prepared PEGMS and PEC. The dissolution behaviors of nonstoichiometric PEC were found to depend on the pH of the solution as well as on the PEGMS/CS composition. The study of MTT assay suggested that the viability of HepG2 human hepatoblastoma cell on PEC were increased significantly in accordance with mole ratio of CS. As the results, the obtained several product is a useful intermediate, which permits further chemical modification for the amino group of CS and may have potential applications in biocompatible or cosmetic systems. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 3057–3070, 2007

Key words: stoichiometric and nonstoichiometric polyelectrolyte complex; chitosan; polyethyleneglycol-monosuccinate; biocompatibility; cell viability

INTRODUCTION

The term polyelectrolyte is employed for polymer systems consisting of a macroion, i.e., macromolecule carrying covalently bound anionic or cationic groups and low-molecular counterions securing for electroneutrality.[1] Macromolecular complexes of different polymers are bound through intermolecular interactions, such as hydrogen bonding, coulomb forces, Vander Waals forces, and transfer forces. Polyelectrolyte complex (PEC) was prepared by the formation of complexes from the interaction of oppositely charged polymers.[2] The mechanism and properties of polymer complexes depend on the charge ration of anionic-to-cationic polymers, the degree of neutralization, flexibility, functional group structure, charge density, stereo regularity, and temperature.[3] PEC have been applied for stabilizers, thickeners, gelling agents, superabsorbance, flocculants, coating or PEC membranes for special separation processes or microencapsulation. The combination of the polymer and electrolyte character produces new materials with unique properties. At present, PEC is used as selective membranes,[4] capsules and fibers,[5,6] biomaterials,[7] etc. PEC can be prepared in various forms such as a film and a hydro-gel, a microcapsule or a sponge, which can be used as a scaffold in tissue regeneration studies. The effects of PEC films composed of polysaccharides on cell behavior have been also studied, and have already reported that PEC can stimulate differentiation of osteoblasts and periodontal ligament fibroblasts.[8–10]

Chitosan (CS), which has a biodegradable, nontoxic and antibacterial properties, as well as renewable resource, is the second most widespread biopolymer

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on earth after cellulose and is composed of poly[β-1,4-2-amino-2-deoxy-D-glucopyranose] repeating units.[11–12] Chemical modification of CS to generate new biofunctional materials is of prime interest because the modification would not change the fundamental surface of CS, would keep the original physicochemical and biochemical properties and finally would bring new properties depending on the nature of the group introduced.[13] Since CS itself is nontoxic and biodegradable[14–16] and shows widespread biological activities,[17–20] it is an appealing bioactive polymer for further development. Unfortunately, its poor solubility in both organic solvents and aqueous solutions has hampered its widespread development. Recently, this problem has been partly overcome by using counteranions of organic acids[21,22] and chemical modification of CS has been carried out. However, to obtain good solubility in water, a large number of hydrophilic groups must be introduced to provide CS derivatives where most of the glucosamine units are modified. To obtain a highly water-soluble CS derivative by a low degree of substitution, a high-molecular weight hydrophilic modifier is preferable. However, there are almost no reports on the modification of CS using polymers.

Among the water-soluble polymers, polyethylene glycol (PEG) is one of the most interesting what is because of its potential medical applications. The water soluble and non toxic PEG is amphipathic polymer frequently used as pharmacological product showing hydrophilicity, biocompatibility, and nonimmunogenicity.[23,24] with low biodegradability. PEG has been used extensively for the modification of biomaterial surfaces in attempts to impede protein adsorption and cell adhesion.[25,26] Surface modification with PEG has been carried out by various methods, for example, simple physical absorption, chemical coupling, and graft polymerization.[27] However, PEG has somewhat of the disadvantage of low chemical reactivity with the bioactive polymer at a low temperature.

Moreover, PEC possesses several properties, which make it a feasible carrier molecule for the preparation of macromolecular derivatives of bioactive agents. However, this polymer has somewhat of the disadvantage of low chemical reactivity with the bioactive compound at a low temperature. A route to solve this problem may be the creation of the more reactive functional group in its structure that enables the coupling with other compounds. In this respect, we confirmed the formation of PEC by interaction between amino group of CS and PEGMS groups that were previously linked to PEG.

This study suggests that the PEC can be used as a biomaterial for repairing or regenerating tissues. In addition, because the PEC is composed of polysaccharides, PEC is expected not to elicit immune responses against it and to have better biocompatibility with the human body, although this is yet to be proved. The most hydrophilic polymer materials are ion-exchange materials whose important component is polyelectrolyte with strongly ionized functionalities. To avoid the membrane dissolution, the macromolecular ionic materials must be stabilized crosslinking. This crosslinking is achieved by covalent linkages between the polymeric chains, by grafting a polyelectrolyte onto an insoluble polymer, by coulomb binding via polyvalent counter-ions, or by the formation of insoluble but swell-able polyanion-polycation complexes.[28] So far, various approaches have been published on the synthesis of PEG-CS derivative. For example, Amiji reported on the synthesis of anionic PEG derivative for the modification of the CS surface in blood contacting applications.[29] Aiba also studied the synthesis of PEG-CS derivatives by reacting cyanuryl trichloride activated mono-methoxy PEG and monomethoxy polyethylene glycol-succinimidyl succinate with amino group of CS.[30] Grafting PEG onto CS is considered to be a convenient way to water-soluble CS derivatives and to be synthesized for instance new bio-functional materials such as drug carriers of anticancer.[31] Although PEG grafted with CS is lightly soluble in water, PEG-CS could not dissolve easily in aqueous solution and it also could not be observed the transmittance. Therefore, in the present study, to create of new more reactive and stable functional group in PEG chain, monosuccinate was picked as materials and the CS surface was modified by the complex method supplying an anionic PEGMS under the appropriate conditions.

We have focused on both the preparation of water-soluble, biocompatible of PEC and establishment of best condition in a novel stoichiometric and nonstoichiometric PEC formed by interaction between PEGMS and CS. In addition, as the PEC is composed of polysaccharides and offers water-solubility, it enables the coupling of the bioactive agent and the application to cosmetic and biomedical agents by formation of salt with CS.

EXPERIMENTAL

Materials

CS (water content maximum 10%) derived from crab shell was a commercial powder kindly supplied by Taehoon Bio, Korea. To increase the degree of deacetylation, the following treatments were applied: A 1% CS solution was prepared by adding 10 g of CS powder in 1 L of 1% (v/v) aqueous acetic acid solution by stirring and this aqueous CS/acetic acid solution were added drop-wise into 40% (w/w) NaOH solution. The CS solutions were filtered through a glass filter and then rinsed free of alkali with distilled

water repeatedly. After this treatment, its weight average molecular weight (M_w) was found to be 31,000 and the degree of deacetylation was determined to be 96.5% by the conductometric method for colloidal titration. Polyethylene glycol (PEG, $M_n = 300$), succinic acid (SA), and succinic anhydride ($M_n = 100$) with 99.9% + purity level were purchased from Aldrich, USA. The water used in the following procedure was obtained from distilled water. All other commercially available solvents and reagents were of analytical grade and used without further purification.

Preparations of stoichiometric and nonstoichiometric polyelectrolyte complex

Preparations of stoichiometric polyelectrolyte complex

Preparations of polyethylene glycol-monosuccinate. To synthesize PEGMS, 30 g of PEG, and 10 g of succinic anhydride were mixed with 1 : 1 mol ratio in solution states and the mixture was reacted at 60°C for 6 h with stirring. After the required time, the PEGMS yielded was maintained for ~ 1 h at room temperature to ensure homogenization. The characterization of PEGMS was carried out by measurements of Fourier transform infrared (FTIR). Following spectral data shows the example for PEGMS with stoichiometric ratios. Selected data for PEGMS: IR: ν 3462 (OH), 1735 (overlab carboxylic acid and ester C=O), 1464 and 1348 (CH_2 band), 1132 (ester C—O). ^{1}H-NMR (D_2O): δ (ppm) 2.54, 2.57, and 2.61 (m, succinate $OCOCH_2CH_2$), 3.50−3.67 (s, PEG OCH_2CH_2OCO), 4.16 (w, broad, OH). ^{13}C-NMR (D_2O): δ (ppm) 31.48 ($OCOCH_2CH_2$), 63.15, 66.71, 71.21, 72.27, 72.40, and 72.50 (CH_2OCO and CH_2OCH_2), 74.57 ($HOCH_2$), 176.88 (ester C=O), 178.65 (carboxylic acid C=O). Anal Calcd for $(C_{14}H_{30}O_8)_{0.5}(C_4H_4O_3)_{0.5}$: C, 50.70; H, 7.98. Found: C, 49.85; H, 7.71.

Preparations of SACS. For comparison with PEC solution dissolved by PEGMS, SA (2.93 g) was dissolved in 93.07 mL of distilled water with stirring at 60°C for 1 h. After the temperature of dissolved aqueous solution in the SA had cooled to room temperature, CS (4 g) was added into the aqueous SA solution. The mixed solution was stirred at ambient temperature for 2 days. After this reaction, the solution was filtered and spread on a clean glass plate and dried in a vacuo at 40°C overnight. Selected data for SA-CS: IR: ν 3375 (NH_2 symmetric stretching vibration), 1727(ester C=O), 1686 (amide I), 1556(NH_2), 1307 (amide III), 1089 and 920 cm^{-1} (saccharide). ^{1}H-NMR (D_2O): δ (ppm) 2.35 (SA CH_2CH_2), 2.96 (H-2), 3.52−3.69 (H-3, H-4, H-5, H-6), 4.89 (H-1). ^{13}C-NMR (D_2O): δ 31.88, 32.25 and 32.60 (SA CH_2CH_2), 58.52 (C−2), 62.52 (C−)6, 72.45 (C−3), 77.44 (C−5), 79.00 (C−4), 100.26 (C−1), 180.59 (NH_3^+ $^-$OOC).

Preparations of PEG-SACS. PEG (7.44 g) was added into the above prepared SACS solution and stirred gently at room temperature for 2 days. The completely mixed solution was filtered using a glass filter and spread on a clean glass plate. The product was vacuum dried overnight at 40°C. This following spectral data shows the characterization of PEG-SACS by FTIR.Selected data for PEG-SACS: IR: ν 3417 (OH and NH), 1732 (ester C=O), 1653 (amide I), 1563 (NH_2), 1350 (amide III), 1099 and 944 cm^{-1} (saccharide). ^{1}H-NMR (D_2O): δ (ppm) 2.34 (SA CH_2CH_2), 2.95 (H-2), 3.43-3.53 (PEG CH_2CH_2), 3.62-3.71 (H-3, H-4, H-5, H-6), 4.89 (H-1). ^{13}C-NMR (D_2O): δ 32.16 (SA CH_2CH_2), 58.92 (C−2), 63.10 (C−6), 63.36, 72.26, and 74.43 (PEG CH_2CH_2), 73.65 (C−3), 78.12 (C−5), 79.24 (C−4), 100.84 (C−1), 180.20 (NH_3^+ $^-$OOC).

Preparations of PEGMS-CS. The PEGMS (9.92 g) obtained from above reaction was added into 86.08 mL of the distilled water and the mixture was stirred vigorously with a magnetic stirrer at room temperature for 30 min to completely the dissolution. The aggregated PEGMS solution was fully dissociated, after which it could by easily dissolved in the water for 30 min. To 1 : 1 mol reaction, the CS (4 g) was dissolved in above aqueous solution with stirring and the reaction was continuing at room temperature for 2 days. In the total volume, the concentration of used PEGMS and CS was controlled at about 13%. The prepared solution was maintained for 1 h to ensure homogenization. This prepared solution was filtered using a glass filter and then it was cast in a clean glass plate, and left additional 24 h to form films of PEC in a vacuum at 40°C. Following spectral data shows the characterization of PEGMS-CS by FTIR. Selected data for PEGMS-CS: IR: ν 3427 (OH and NH), 1733 (ester C=O), 1656 (amide I), 1569 (NH_2), 1387 (amide III), 1097 and 947 cm^{-1} (saccharide). ^{1}H-NMR (D_2O): δ (ppm) 2.38–2.54 (m, succinate $OCOCH_2CH_2$), 3.02 (H-2), 3.34(3.67 (s, PEG $OCOCH_2CH_2$), 3.71–3.79 (H-3, H-4, H-5, H-6), 4.07 (w, broad, OH), 4.96 (H-1). ^{13}C-NMR (D_2O): δ 32.93, 34.30, and 35.95 ($OCOCH_2$), 58.16 (C-2), 63.06 (C-6), 63.26, 66.55, 71.16, 72.12, and 74.40 (asymmetric OCH_2CH_2O), 73.16, 76.95 and 79.09 (C-3, C-5, C-4), 100.26 (C-1), 178.31–183.25 (OCO and NH_3^+ $^-$OOC). Anal Calcd for $(C_6H_{10}N_1O_4)_{0.065}$ $(C_{18}H_{34}O_{11})_{0.065}0.87H_2O$: C, 6.22; H, 11.04; N, 0.57. Found: C, 6.20; H, 11.12; N 0.52.

Preparations of nonstoichiometric polyelectrolyte complex

As prepared above, a given amount of solution of PEGMS and a known weight of purified CS powder was slowly added to 88 wt % distilled water at room temperature with stirring. Table I summarized the composition of CS and PEGMS used for preparing various PEC samples. The compositions of the CS/

TABLE I
The Charged Composition According to Mixing Ratios of CS and PEGMS

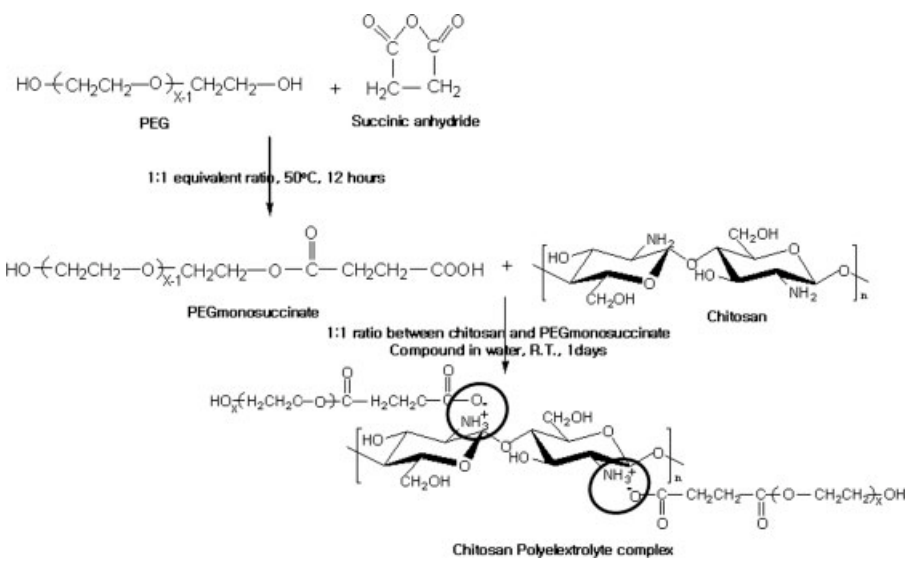
Component (g)	PEC 5/95	PEC 10/90	PEC 15/85	PEC 20/80	PEC 25/75	PEC 30/70	PEC 35/65	PEC 40/60	PEC 45/55	PEC 50/50
CS	0.6	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6
PEGMS	11.4	10.8	10.2	9.6	9.0	8.4	7.8	7.2	6.6	6
Water	88	88	88	88	88	88	88	88	88	88
Equivalent Ratio	1/6.118	1/2.898	1/1.824	1/1.288	1/0.966	1/0.751	1/0.598	1/0.483	1/0.394	1/0.322

PEGMS blends were stepwise controlled by adjustment of various mixing ratio by weight. For example, PEC40/60 represents the PEC containing 40 wt % CS and 60 wt % PEGMS in concentrations of added 12%. The completely suspended solution of PEGMS and CS added in distilled water was obtained from mechanical stirring for 2 days at room temperature. Subsequently, the mixed solution was filtered through a glass filter to remove undissolved impurities and then the filtered solutions were prolonged for 24 h to form solids states *in vacuo* at 40°C. Nonstoichiometric PEC compounds were prepared by varying the ratio of CS to the PEGMS components. The PEC compounds with CS : PEGMS, in 5 : 95 (PEC5/95), 10 : 90 (PE10/90), 15 : 85 (PEC15/85), 20 : 80 (PEC20/80), 25 : 75 (PEC25/75), 30 : 70 (PEC30/70), 35 : 65 (PEC35/65), 40 : 60 (PEC40/60), 45 : 55 (PEC45/55), and 50 : 50 (PEC50/50) ratio were prepared and characterized, respectively. From the analysis of the experimental section, all the schematic procedures followed here for synthesis the PEC was showed in Scheme 1.

Characteristics

For study on chemical characterizations, The C, H and N elemental analysis was performed with FISONS EA1108 analyzer. FTIR spectra were recorded on FTIR (Bruker IFS-66v/s, Germany) spectrophotometer to observe the chemical structure of various compounds in the spectrum range of 4000–800 cm^{-1}. ^{1}H and ^{13}C-NMR analysis of each compounds dissolved in D_2O were carried out using FT-NMR (Bruker DPX-300, Germany) spectrometer at 300 MHz.

For study on physical characterizations, UV spectra of the prepared samples in liquid states were obtained on a Varian Cary 500 UV-Visible spectrometer. Each spectrum was scanned over the range of 200–400 nm at a speed of 10 nm/min. Data were collected and plotted using a UVPC program and computer data station supplied by the manufacturer. Diffractograms using a Wide angle X-ray diffractometer (WAXD, Rigaku D/max-2500) were obtained for the compounds. WAXD patterns were recorded by the reflection method with nickel filtered Cu Kα radiation (wavelength 1.54 E) operated at 40 kV, 30 mA in the 2θ scanning mode between 5° and 40°. The thermal characteristics of the sample was analyzed using a differential scanning calorimeter (DSC, Perkins Elmer thermal analysis system) cooled with liquid nitrogen circulation. Sample (accurately 5 mg) was prepared after conditioning and sealing this into aluminum



Scheme 1 Synthetic route of PEC.

pans. The pan was placed in a stage and heated from −40°C to +240°C with 10°C/min of heating rate and 50 mL/min of nitrogen gas flow rate. The cross-section morphological structure of samples was observed through a scanning electron microscope (SEM, Hitachi *S*-4200) instruments. The samples were sputter-coated with a layer of white gold (400 Å. The fractured cross surfaces of samples were achieved by cooling in liquid nitrogen. All images were taken at 20 kV power and 1000× magnification. The solubility test of the prepared compounds was evaluated in aqueous media at a wide range of pH. The results of the solubility test were summarized in Table II. The pH of nonstoichiometric solid formable PEC was recorded using a digital pH meter (Metter-Toledo GmbH Schwerzenbach, Switzerland) and also the viscosity was measured by Brookfield viscometer (DV-1, Brookfield Engineering Labs. Inc, USA) using a LV spindle (spindle number 3 or 4) under the 60 rpm of turning speed of spindle at room temperature.

For study on biological characterizations, a HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA) for biocompatibility test of the stoichiometric and nonstoichiometric PEC. In culture of cells, the HepG2 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 200 IU/mL of penicillin, 200 μg/mL of streptomycin and 1 m*M* of sodium pyruvate. The culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization. Subsequently, cell viability assay was estimated by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodium bromide) staining method. Cells from 4 to 5-day-old cultures were seeded in 24-well plates at the density of 2×10^4 cells/well. The volume of the medium in the wells was 1 mL. In control experiments, cells were grown in the same media containing a drug-free vehicle. After incubation with the drug for 48 h, 100 mL of MTT (5 g MTT/L in H_2O) were added to each well and then cells incubated for a further 4 h. DMSO (500 μl) were added to each culture and mixed by pipette to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with an ELISA reader (Molecular devices, Menlo Park, CA) with a 540 nm filter.

TABLE II
Solubility of Each Compound at a Wide Range of pH

Compound	Acetic acid solution (1%)	Distilled water	NaOH solution (1%)
SA-CS	Soluble	Soluble	Swelling
PEGSA-CS	Soluble	Soluble	Swelling
PEGMS-CS	Soluble	Soluble	Swelling

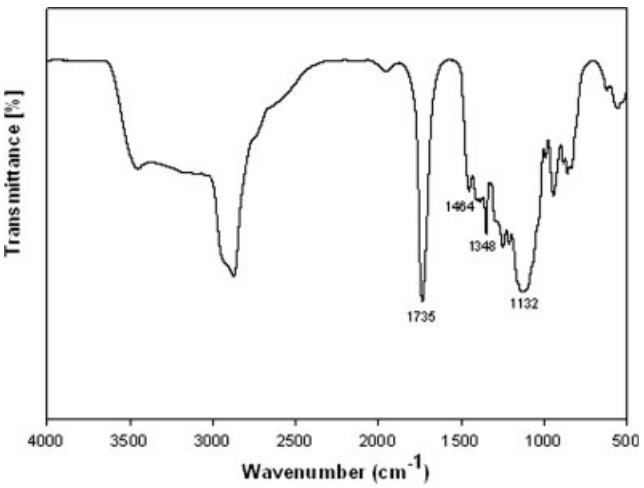


Figure 1 FTIR spectra of synthesized PEGMS.

RESULTS AND DISCUSSION

Characteristics of stoichiometric polyelectrolyte complex

FTIR and ^{1}H and ^{13}C-NMR spectra of PEGMS

To show evidence of specific interactions, the formation of PEGMS by esterification between MS and the end group of PEG was confirmed by FTIR analysis (Fig. 1) and also characterized by ^{1}H and ^{13}C-NMR spectroscopy at room temperature in D_2O (Fig. 2). As seen in Figure 1, the presence of formed ester group (C=O stretching peak) by synthesis between PEG and succinic anhydride was first ensured from the strong absorption band at 1735 cm^{-1} and the adsorption of hydroxyl group is the wide adsorption band observed ranging ~ 3462 cm^{-1}.

In ^{1}H-NMR (a) spectra of Figure 2, the PEGMS was shown in signals at δ 2.49–3.68 ppm, which corresponds to methylene protons in the main chain of PEGMS. The multiplet signals were attributed to structure of asymmetric PEGMS caused by formation of the ester group in Figure 2. Also, the signals at around δ 2.4 ppm were multipleted by same reason. In Figure 2(b), the ^{13}C-NMR spectra of the PEGMS complex were confirmed that the signals of methylene carbon atoms of synthesized PEGMS in comparison with the signals of PEG showed more multipleted signals at δ 63.2–74.6 ppm. This multiplet is applicable to methine carbons of asymmetric PEGMS caused by formation of the ester bond. Furthermore, two bands appeared singlet at δ 176.9 and 178.7 ppm were indicated carbonyl ester and carboxylic acid groups corresponding to the monosuccinate group, respectively. These results indicated that the esterification reaction was undoubtedly completed between PEG and succinic anhydride by synthesis.

Structural analysis of stoichiometric PEC

Since the amino group has stronger nucleophile than the hydroxyl group, the synthesized PEGMS can be

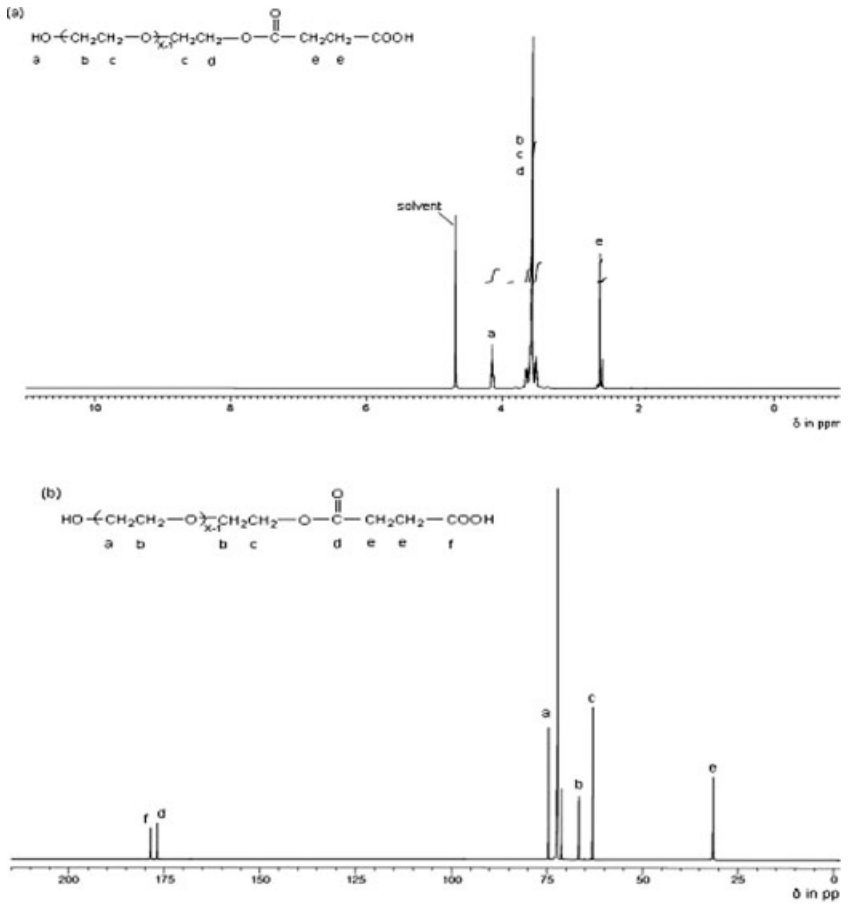


Figure 2 ^{1}H-NMR (a) and ^{13}C-NMR (b) signals of PEGMS in D_2O.

chosen as the complex group with the amine group of CS and easily be introduced to the CS. Then, formed PEC by interaction between two compounds with high-molecule weights was carried out. All the reactions proceeded with smoothly in a solution states (Scheme 1). Figure 3 shows the FTIR spectra of SACS (a), PEG-SACS (b) and PEGMS-CS (c). In the case of PEGMS-CS, unlike SACS or PEG-SACS, it was found that distinctive absorption bands appeared at 1656 and 1387 cm^{-1} that can be attributed to the formation of an amide I bond and amide III characteristic amino peak. In addition, the absorption bands at 1097 and 947 cm^{-1} peaks assigned to the saccharide structure, and also a new absorption peak at 1569 cm^{-1}, which did not appear in SACS and PEG-SACS could be identified the formation of an characteristically strong amino peak of CS. Compared with those of PEGMS-CS, the FTIR spectra of SACS and PEG-SACS showed absorption signals at 1556 and 1562 cm^{-1} assigned to the characteristic peaks of amino groups, but the absorption peaks recorded amide I and III band were shown in indistinct appearance.

It is previously seen in Figure 2 that the formation of PEGMS between PEG and MS was examined by ^{1}H-NMR and ^{13}C-NMR to show evidence of specific interactions. The proton and carbon NMR spectra of PEGMS ensured the presence of the formed ester group by synthesis between PEG and MS. The proton NMR spectrum [Fig. 2(a)] showed that the multiplet signals around δ 2.49 and 3.68 ppm were attributed to structure of asymmetric PEGMS caused by formation of the ester group. And also the signals at around δ 70

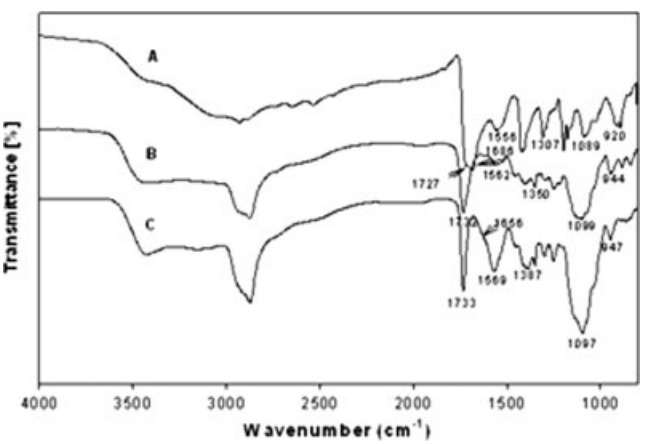


Figure 3 FTIR spectra of stoichiometric PEC: (A) SA-CS, (B) PEG-SACS and (C) PEGMS-CS.

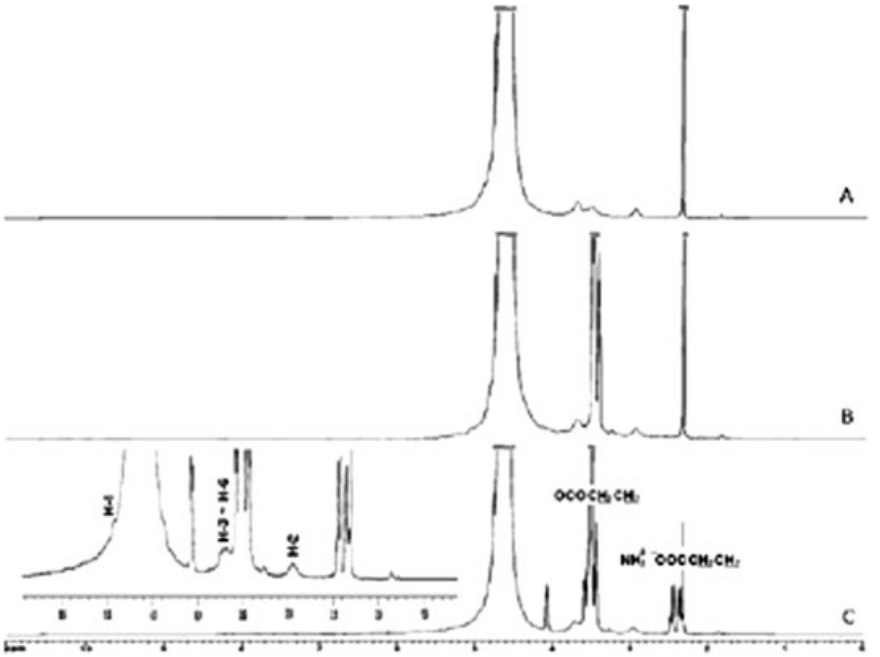


Figure 4 ^{1}H-NMR Signals of the prepared compounds in D_2O: (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

and 176 ppm in the ^{13}C-NMR [Fig. 2(b)] were assigned that structural change caused by formation of the ester group. These results indicated that the esterification reaction was certainly completed on synthesis between PEG and MS. Figures 4 and 5 were showed the ^{1}H and ^{13}C-NMR spectra for SACS, PEG-SACS and PEGMS-CS. As shown in Figures 4 and 5, it was found that the stronger proton and carbon NMR peaks could be obtained for PEGMS-CS vibration than SACS and PEG-SACS ones. Some evidence signals about the prepared stoichiometric PEC by interaction between PEGMS and CS compounds were assigned by the ^{13}C-NMR signals [Fig. 5(c)] from about δ 178.31 to 183.25 ppm. In addition, ^{1}H and ^{13}C-NMR spectra of PEGMS-CS showed that more split signals than SA-CS and PEG-SACS signals were attributed to asymmetric structure caused by preparation of the stoichiometric PEC, whereas the PEG-

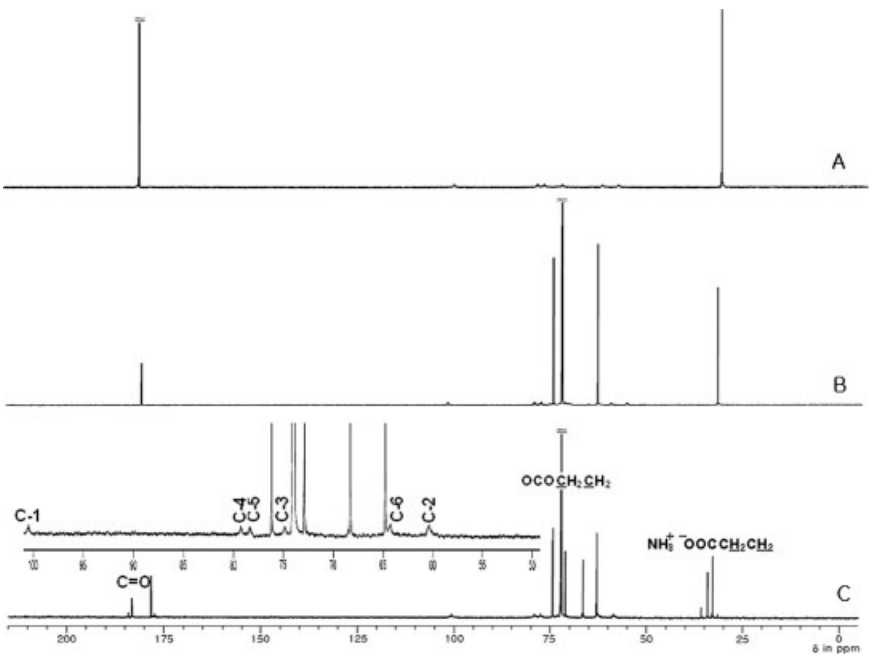


Figure 5 ^{13}C-NMR Signals of the prepared compounds in D_2O: (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

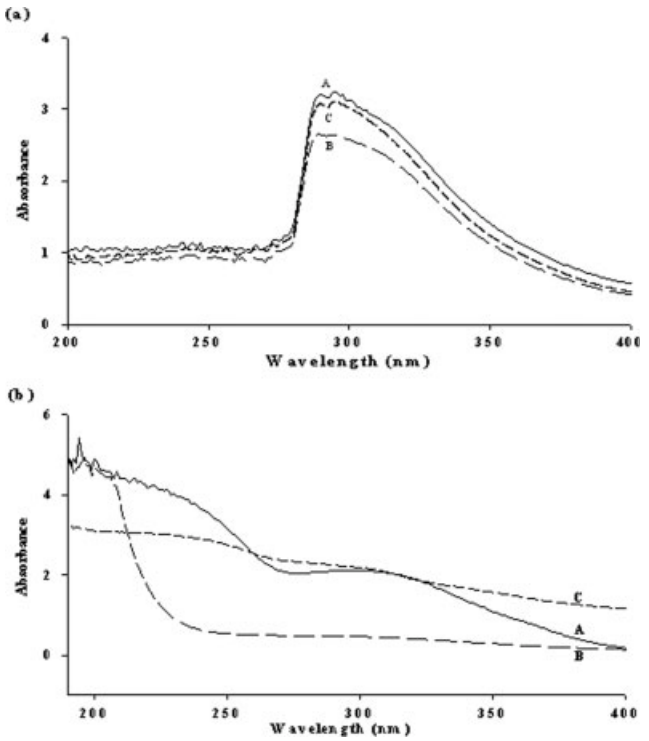


Figure 6 UV-VIS absorbance of each compound in the solution state (a) and film state (b): (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

SACS signals were indicated that SACS and PEG were just mixed different from signals of PEC which it was synthesized by electrostatic interaction between two compounds.

The comparison of the UV-Visible absorption of SACS and PEG-SACS and PEGMS-CS in the solution (a) and film state (b) is shown in Figure 6. In the Figure 6(a), the UV-Visible absorption spectra in the solution state of SA-CS and PEGSA-CS compound revealed almost the same absorption spectra in the 280–380 nm of PEGMS-CS, accompanied with the wavelength very close to each compound. But, the data of SACS solution shows higher absorbance than PEGMS-CS, it probably because of SA has more carbonyl functional group than PEGMS. However, the PEGMS-CS solution shows higher absorbance than PEG-SACS. It suggested that prepared PEGMS-CS by interaction between two polymers has higher absorbance than just mixed PEG-SACS. In Figure 6(b), the measured absorption in the film state showed that PEGMS-CS appeared broader and higher absorbance than SACS and PEG-SACS. This increase of absorption and wavelength is due to strong interaction between two polymers.

The crystalline characteristics of SACS as well as PEG-SACS and PEGMS-CS were determined by X-ray diffractometer and the diffraction patterns were shown in Figure 7. It has been generally known that the X-ray diffraction scan of CS exhibits three reflection falls at $2\theta = 11^\circ$, $2\theta = 20^\circ$, $2\theta = 22^\circ$ indicating the crystalline region of CS. Samuels.[32] reported that the reflection fall at $2\theta = 11^\circ$ was assigned to crystal forms I and the strongest reflection fall at $2\theta = 20^\circ$ which corresponds to crystal forms II. However, in Figure 7(c), It is seen that there is only one broad peak at around $2\theta = 20^\circ$ which the diffractograms of PEC show clearly difference from that of SACS (a) and PEG-SACS (b) form. No peak is found except at around 20° in the diffractograms of PEC. This inferred that its ability to form a hydrogen bond might be increased by reaction between PEGMS and CS compounds. In addition, the stoichiometric PEC does not crystalline form in reaction and maintains amorphous states during blends. After compared PEGMS-CS with SACS and PEG-SACS, it showed reflection falled at around $2\theta = 12^\circ$ and $2\theta = 18^\circ$. This may prove that any bond by interaction between two components not happened and the two compounds just were mixed with crystalline form of SA.

The thermal transition of SACS, PEG-SACS and PEGMS-CS were determined by DSC analysis and showed in Figure 8. The scan temperature was raised from −40°C to 240°C, ensuring each compounds were decompose. The thermal behavior of SACS is different from that of PEG-SACS as well as PEGMS-CS as can be observed in Figure 8. In three spectra, CS dissolved in succinic anhydride (SA-CS) shows only a significant transition at around 190°C (c) while PEGSA-CS has two bolder decomposition level with one starting at around 80°C and another starting at around 190°C (b). Concerning the transition temperature of CS, it has been reported previously several authors, among them, Sakurai et al.[33] reported the glass transition of CS was found to be 203°C determined by DSC. In most recently, Suyatma et al.[34] found the glass transition of CS is observed at 194°C,

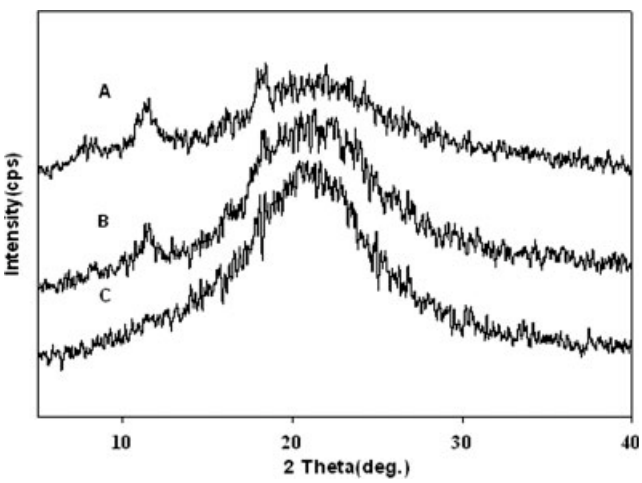


Figure 7 WAXD patterns of each compound: (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

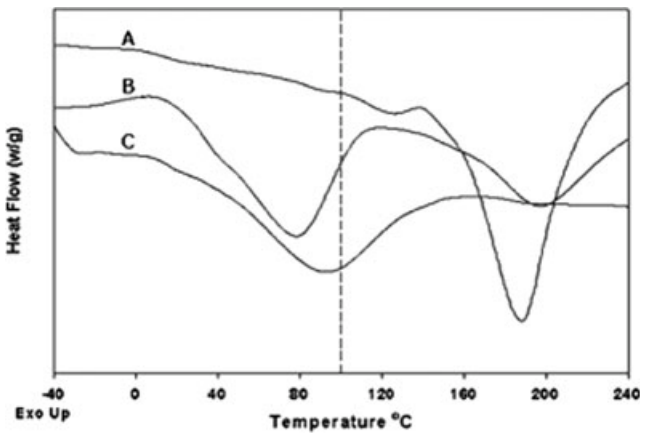


Figure 8 DSC thermograms of each compound: (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

close to that of the SACS in our present study. The thermal transition curve of PEGMS-CS (c) shows a higher and broader decomposition temperature than just mixed PEG-SACS (b). The former endothermic transition may be due to the melting point of PEG and the some loss of moisture content in the CS. The endothermic peak of PEGMS-CS is relatively higher and broader than the result of PEG-SACS. This could be attributed to the fact that formation of complex caused by interaction between PEGMS and CS. On the other hand, from an endothermic peak at around 180°C, the results indicated that two compounds are just mixing like to show in the PEG-SACS curve. That is to say, PEGMS-CS complex exhibit higher decomposition temperature when compared to the PEG-SACS compound. This variation in thermal events proved the PEC compound formation. In addition, this result demonstrated that the higher decomposition temperature of PEGMS-CS complex was enhanced thermal stability.

The SEM images of the surfaces of three compounds described in Figure 9. The cross surfaces of each complex film was exhibit uneven or uniform microstructure. The SACS film showed scale-like and irregularly thickened microstructure. On the other hand, the PEG-SACS film shows quite rough surfaces such as nonuniformity particles immersed in a matrix that may be due to the two components between PEG and CS existed in as a separated phase and also aggregation phenomenon was clearly distinguished them from the PEGMS-CS complex. The absence of uniformity of SACS and PEG-SACS is obvious in SEM. The poor complex of SACS and PEG-SACS has lead to the uneven or irregular formation of compounds. In contrast, the surface morphology of PEGMS-CS complex was exhibit remarkably homogeneous and dense structure. As for this result of cross surfaces, a possible reason may be that PEGMS were formed with CS by strong electrostatic interaction. Therefore, the PEGMS-CS complex film was shown as smoother than the only blended two components.

Table II lists the solubility of SACS, PEG-SACS and PEGMS-CS compound in distilled water, aqueous acid and alkali solvents. After stirring in room temperature for 24 h, the three compounds were dissolved in the acetic acid/water solution and the distilled water but did not dissolve in NaOH solution completely. In the acidic media, each compound was well dissolved but swelled in the alkaline media. This result should be taken into account that the complex between carboxyl groups of PEGMS and amine groups of CS occur, resulting in electrostatic interaction of two polymers.

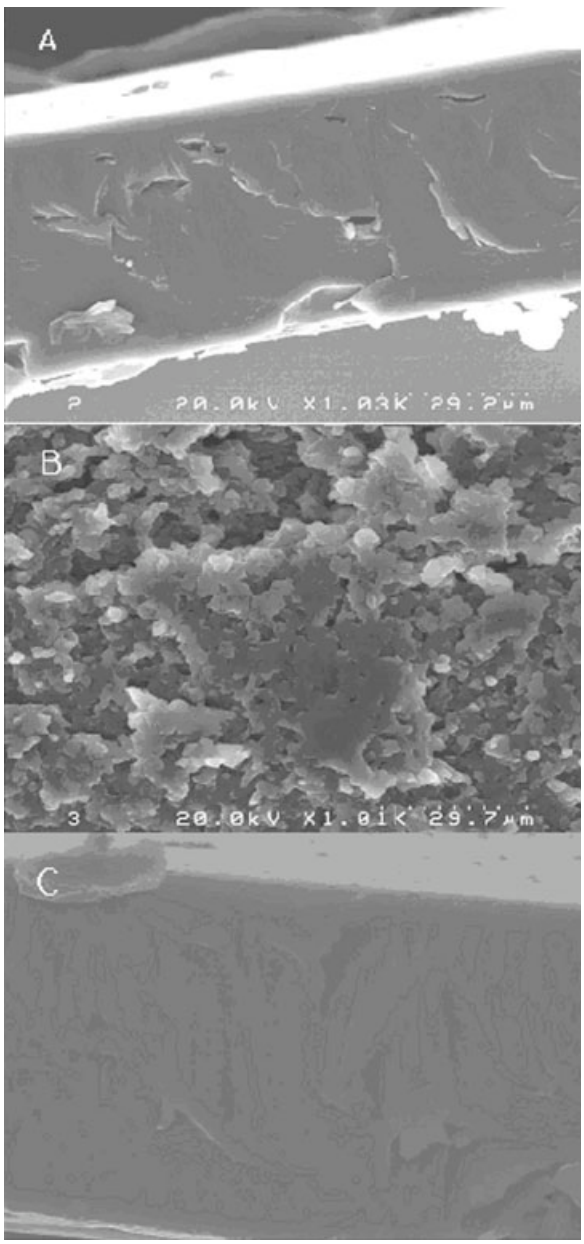


Figure 9 Cross surfaces of each complex film: (A) SACS, (B) PEG-SACS, (C) PEGMS-CS.

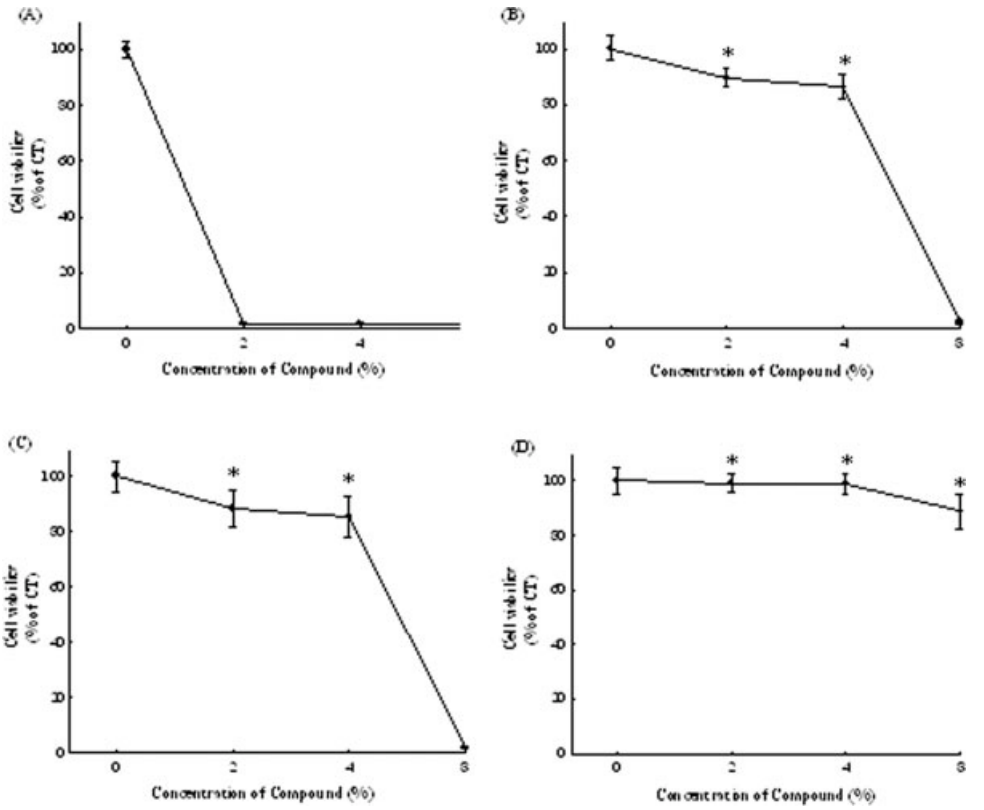


Figure 10 The effect of each compound on cell viability in HepG2 human hepatoblastoma cells: (A) PEGMS, (B) SACS, (C) PEG- SACS, (D) PEGMS-CS.

Biocompatibility and cytotoxicity of stoichiometric PEC

Cell viability test by cell growth is a proper method to determine the biocompatibility of the biomaterials. The effect of each compound (PEGMS, PEGMS-CS, SACS, and PEG-SACS) on the viability of HepG2 cells was examined using a MTT staining method to assess the biocompatibility of the stoichiometric PEC. MTT assay was used as a measure of relative cell viability. Figure 10 displays the cell viability that was dependent on the increase in the total concentrations of each compound. PEGMS showed significant cytotoxicity to all the concentrations tested of 2, 4, and 8%, while that of the SACS and PEG-SACS was observed above 4% concentration. However, the stoichiometric PEC was noted no significant decrease in cell viability up to the concentration of 8%, assuming the most cells still kept viability and only some cells inactivate. It can therefore be concluded that the stoichiometric PEC is highly biocompatible and does not observed significant cytotoxicity. It also denotes that the compound is possibility has a biomedical application.

Characteristics of nonstoichiometric polyelectrolyte complex

Dissolution behavior and phase transition phenomenon

The dissolution behaviors of CS immersed in PEGMS solution and the phase transition phenomenon from liquid state to solid state for PEC5/95, PEC10/90, PEC15/85, PEC20/80, PEC25/75, PEC30/70, PEC35/65, PEC40/60, PEC45/55, and PEC50/50 of prepared nonstoichiometric PEC were shown in Table III. The dissolution procedure was estimated from the

TABLE III
Dissolution Behavior of CS and the Phase Transition Phenomenon of Nonstoichiometric PEC According to Mole Ratios of CS versus PEGMS

Dissolution and film formation	PEC 5/95	PEC 10/90	PEC 15/85	PEC 20/80	PEC 25/75	PEC 30/70	PEC 35/65	PEC 40/60	PEC 45/55	PEC 50/50
Dissolution of CS in PEC solution	D	D	D	D	D	D	D	D	ND	ND
Solid film formation of PEC	L	L	L	LS	S	S	S	S	–	–

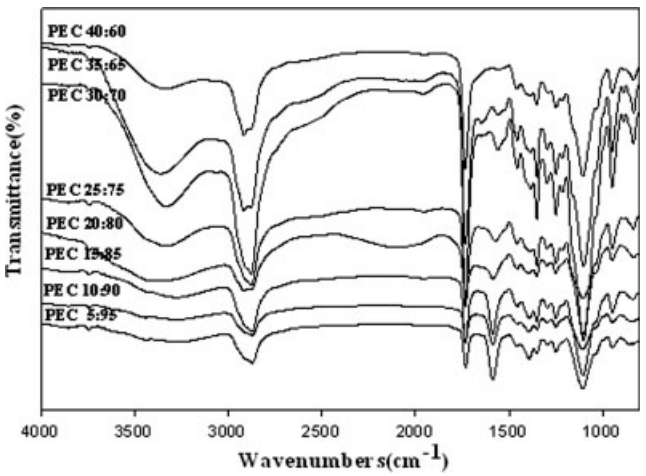


Figure 11 FTIR spectra of various nonstoichiometric PEC compounds.

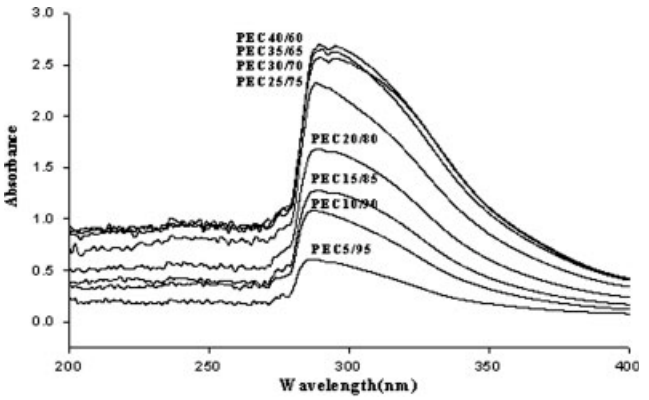


Figure 12 UV spectra of nonstoichiometric PEC according to mixing ratios of CS versus PEGMS. Total concentration of PEC was fixed about 12%.

increase in weight of original CS immersed in PEGMS. The dissolution behavior of CS was found that the CS was well dissolved in PEGMS from 5 to 40 wt % concentrations. However, the dissolution of CS in PEGMS was not permit in the above 45%. In results of this phase transition phenomenon observation, the solid formation of PEC could be possible from PEC25/75, PEC30/70, PEC35/65 and PEC40/60, while PEC5/95, PEC10/90 and PEC15/85 were could not able to form of solid states and also the PEC20/80 was partially formed. It can be strong supported that the PEC film can be cast from 20 to 40 wt % concentrations. That is, the possible amount for film formation was the minimum when CS: PEGMS were mixed in 20 : 80 ratios.

Structural analysis of nonstoichiometric PEC

Figure 11 shows the FTIR spectrum of eight nonstoichiometric PEC from different complex formation performs using different ratios of CS and PEGMS. The spectrum of all the PEC compounds was quite similar as can be seen.

It was exhibit that the most distinctive absorption bands appeared at 1387 and 1656 cm^{-1}. This characteristic 1387 cm^{-1} peak were attributed to CH_3 in symmetric bending of amide I contained in the each PEC film. The other carbonyl peak (C=O) in PEGMS was observed at 1656 cm^{-1}, which was the result of the carbonyl stretch of the carboxylic groups in PEGMS. In comparison with the PEGMS complex as previously seen in Figure 1, the PEC spectrum was observed significantly different absorption peaks in the region of around 1650 cm^{-1}. The peak absorbed at 1650 cm^{-1}, which assigned to a symmetric deformation of NH_3^+,[35] almost disappeared in the spectrum of PEC5/95 and PEC10/90 contained small amount of CS. These results confirmed that the COO^- group in PEGMS was complex with NH_3^+ groups in CS by means of electrostatic attraction. In addition, the strong absorption bands at 1097 and 947 cm^{-1} peaks observed in all PEC compounds were assigned to saccharide structure of CS such as cellulose-ester type absorption, and also the absorption band at 1569 cm^{-1}, which was may be due to the strong amino characteristic peak. All of the solutions were shown in essentially similar peaks. It proves that the series of nonstoichiometric PEC formation achieved successfully.

The UV-VIS spectra obtained from various PEC compounds were shown in Figure 12. The absorption spectra support previous FTIR spectrum. The spectra reveal absorption in the range of 280–300 nm and the PEC40/60 complex shows highest absorbance. This suggests that PEC40/60 was more interacted than others between CS and PEGMS.

pH can have an important effect on the electrostatic interaction between the oppositely charged materials such as PEGMS negatively and CS positively charged substrates. The pH values of PEC40/60, PEC35/65, PEC30/70, PEC25/75, and PEC20/80, which could be solid phase transition, are showed 5.85, 5.32, 5.05, 4.84, and 4.60, respectively, in Figure 13. It was found that the pH value was decreasing with the increasing concentration of the PEGMS used up to a certain extent. The PEC30/70, PEC35/65, and PEC 40/60 were shown in pH above at 5.0. From this result, it is known that the PEC30/70 and PEC35/75 as well as the highest level of PEC40/60, where the pH value is close to that of human skin.

Table IV lists the Brookfield viscosity of PEC20/80, PEC25/75, PEC30/70, PEC35/65, and PEC40/60. It is seen that the viscosity was between 260 and 2510 cp corresponds to increased with increases in the concentration of CS. The viscosity of PEC40/60 is much higher than the PEC20/80 which added more individual component. This result presents that the interpolymer complex system presents high viscosity

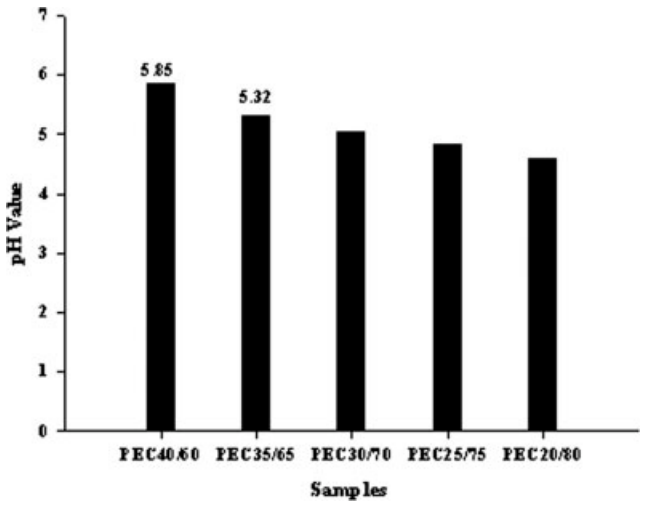


Figure 13 pH value of nonstoichiometric PEC according to mixing ratios of CS versus PEGMS.

behavior caused by the presence of nonstoichiometric component in the systems.

Table V shows the solubility of PEC20/80, PEC25/75, PEC30/70, PEC35/65, and PEC40/60 measured at various solutions of distilled water, aqueous acetic acid and alkali solvents. It was revealed that all the PEC samples were dissolve in the distilled water easily as to form complex. This suggests that two polymers would be complex by interaction between carboxyl and amine group in all samples. All of them were however highly hydrophilic and thus each PEC was well dissolved in the acidic media but swelled upon contact with alkaline media. This behavior may be due not only to the formation of PEC complex but also to the content of CS having a large number of hydroxyl groups. Accordingly, no serious differences can be seen between samples.

Biocompatibility and cytotoxicity of nonstoichiometric PEC

HepG2 human hepatoblastoma cell line was used for biocompatibility assessment for PEC20/80, PEC25/75, PEC30/70, PEC35/65, and PEC40/60. Figure 14 plots the cell viability according to increasing total concentrations of each PEC. In the cases of both PEC20/80 and PEC25/75, the significant cytotoxicity was observed above 4% concentration, while the PEC30/70, PEC35/65, and PEC40/60 were noted no significant decrease in cell viability up to the concentration of 8%. It seems that the HepG2 cells are more active on the polysaccharide surface of PEC compounds according to increasing with CS ratio, irrespective of the total concentrations of PEC compounds. Of course, the cytotoxicity of PEC40/60 is revealed slower than PEC20/80, because of the more amount of CS. The HepG2 cells exhibited compatibility for all concentrations of PEC containing with CS, indicating cells remained viable and the cell was maintained at a comparatively constant level. Thus, no significant cytotoxicity was observed. Consequently, the especially PEC35/65 and PEC40/60 compound, which evidently showed high cell viability, could be considered to be suitable for supporting biocompatible materials in the biomedical and cosmetic applications.

CONCLUSIONS

This study has explained the effect of PEGMS-CS complex. First, the PEC was prepared by interaction between CS and further synthesized PEGMS by esterification of PEG and succinic anhydride. PEGMS can easily be coupled with amines of CS to introduce biofunctionality on PEC films. Subsequently, the PEGMS has been synthesized by stoichiometric reaction and then, the previously prepared PEGMS was treated with CS by nonstoichiometric reaction corresponding with different mole ratios. The chemical structure of PEC was characterized by FTIR and ^{1}H, ^{13}C-NMR spectra, and the structural analysis were performed with UV-VIS spectrum, elemental analysis, X-ray diffraction, and DSC thermograms, and further tested dissolution behavior, phase transition phenomenon, solubility, and cell viability assay. It was found that the observed FTIR characterization confirmed the ionic complex according to interaction of the two polymers and the data was in good agreement with the prepared PEC. Also, it indicates that after the PEC, the CS complex shows good solubility in pure water. In characterization by elemental analysis, ^{1}H

TABLE IV
Viscosity of Nonstoichiometric PEC According to Mixing Ratios of CS versus PEGMS

Sample	Spindle No.	rpm	Toque (%)	Viscosity (cp)
PEC20/80	3	60	13.0	260
PEC25/75	3	60	26.8	536
PEC30/70	4	60	12.0	1200
PEC35/65	4	60	16.0	1600
PEC40/60	4	60	25.1	2510

TABLE V
Solubility of Nonstoichiometric PEC According to Mixing Ratios of CS versus PEGMS

Sample	Acetic acid solution (1%)	Distilled water	NaOH solution (1%)
PEC20/80	Soluble	Soluble	Swelling
PEC25/75	Soluble	Soluble	Swelling
PEC30/70	Soluble	Soluble	Swelling
PEC35/65	Soluble	Soluble	Swelling
PEC40/60	Soluble	Soluble	Swelling

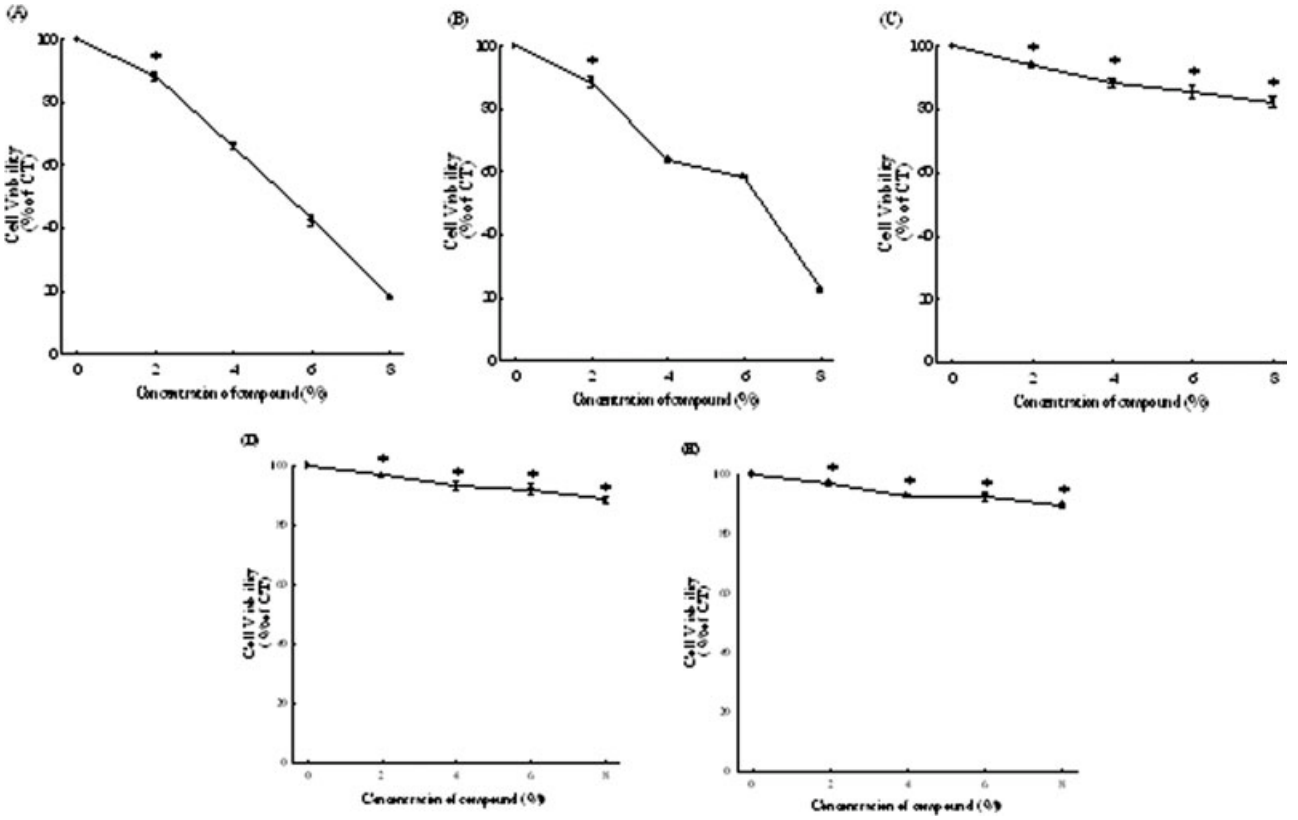


Figure 14 The effects of each compound on cell viability in HepG2 human hepatoblastoma cells. Cells were incubated with or without each concentration of compounds for 48 h. A cell viability assay was done by the MTT staining method. Results are expressed as percent change of the control condition in which cells were grown in medium without compounds. Data points represent the mean values of four replications with bars indicating SEM: (A) PEC20/80, (B) PEC25/75, (C) PEC30/70, (D) PEC35/65, (E) PEC40/60.

and ^{13}C-NMR was revealed adequate evidence that confirmed the chemical structure of PEGMS and PEC. In conclusion, the prepared stoichiometric PEC and nonstoichiometric PEC30/70, PEC35/65, and PEC40/60 has shown no significant cytotoxicity and is especially effective in regards of cell viability of HepG2 human hepatoblastoma cells. As these results, the obtained product is a useful intermediate, which permits further chemical modification for the amino groups of CS and may have potential applications in biomedical or cosmetic systems.

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