Polyelectrolyte Complex (PEC) of the Alginate-Chitosan Membrane for Immobilizing Urease

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Abstract. PEC of the alginate-chitosan membrane as supporting material for immobilizing urease was produced. This study aimed to develop a supporting material for enzyme immobilization that has high stability, a fast response time and an easy and relatively inexpensive preparation procedure. An alginate-chitosan PEC membrane was produced by reacting alginate hydrosol and chitosan (1:1 in mass) at pH 5.28, followed by mixing and drying at room temperature. The FTIR spectra, XRD patterns and SEM assay confirmed that alginate-chitosan PEC was obtained. The color change of the BTB indicator proved that urease was trapped in the cavities of the alginate-chitosan PEC membrane while the immobilized urease still showed catalytic activity. Thus, the membrane of alginate-chitosan PEC has good characteristics as a matrix for urease immobilization.

Keywords: *alginate-chitosan; immobilizing; polyelectrolyte membrane; urease.*

1 Introduction

Enzyme immobilization is carried out by attaching enzymes to or within some suitable support material to improve the stability and reproducibility of the enzyme. In the last decade, various matrixes have been used as enzyme immobilization support. For instance, synthetic polymer membranes [1] and porous silica matrix [2] have good mechanical stability, easy access for enzymes in binding to the active functional groups of the substrate and availability of space when changing geometric configurations. However, they also have limitations, such as incomplete biodegradabality and biocompatibility. Hence, introducing a more environmentally friendly support material for enzyme immobilization like polyelectrolite complex (PEC) membrane is important. The application of PEC membranes as part of an electrochemical

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biosensing system has the advantage of being able to reject signals from species that interfere with analytical measurements, thereby reducing interference currents. This has implications for decreasing the analyte detection limit by increasing the signal-to-noise ratio (S/N) [3].

Polyelectrolyte complexes (PECs) are obtained by mixing aqueous solutions of two polymers carrying opposite charges [4]. PEC formation is due to charge neutralization mainly by electrostatic interactions between polyanion-polycation systems and possible interactions induced by attraction of multivalent counterions between polyelectrolyte segments, i.e. Coulomb forces, hydrogen bonds, transfer forces and van der Waals forces [5]. Alginate as degradable polymer, which is extracted from brown seaweeds (*Phaeophyceae*, mainly *Laminaria*), is a copolymer of α-(1-4)-L-guluronic (G) and β-(1-4)-D-mannuronic acid (M). Alginate as weak polybase has pKa values of 3.38 and 3.65 for M- and G- residues, respectively [6]. Chitosan, as deacetylation product of chitin, is a natural polymer of β-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose monomers. The macro pKa value of this polycationic polymer is in the range of 6.3 to 6.5 [7].

A chitosan aqueous solution is reacted with a polyanion aqueous solution, such as sodium alginate [8]. When dissolved in appropriate conditions, alginate as a polyanion and chitosan as a polycation can interact with each other through the carboxyl group of the alginate and the amino group of the chitosan [9] and its ionic interactions are the main interactions inside the network of PECs. Since electrostatic interactions are present between opposite polyelectrolytes in the formation of PEC, crosslinking agents are not needed. This is expected to minimize the possibility of toxicity and undesirable effects [10]. When functioning as a matrix for immobilizing biomaterials, PEC has an advantage over its original polymers. For this reasons, PEC is a potentially attractive material for application in biotechnology. In this work, the formation and characterization of alginate-chitosan membrane and its application in a urease immobilization matrix is reported. The interaction of alginate and chitosan in the membrane is described and the activity of both free enzymes and immobilized enzymes was determined.

2 Material and Methods

2.1 Chemicals

The urease used for preparing the biosensor was E.C. 3.5.1.5. from jack beans (Type III, U1500), 272 μg was stored at 4 °C, the sodium alginate (300 to 400 cp) was from brown algae, and the chitosan was 95% deacetylated from crab shell purchased from Sigma (St. Lois, USA). Hydrochloride acid (37%), glacial

acetic acid (98%), sodium hydroxide and Bromothymol blue (BTB) were received from Merck (UK). A stock solution of urea (1000 μg/mL) was prepared in aqueous solution using deionized water.

2.2 Preparation of Membrane

The membrane was prepared by mixing two polymer solutions consisting of chitosan hydrosol and alginate hydrosol according to the method from Hermanto, *et al.* [11]. Chitosan hydrosol was prepared from 1 g of chitosan that was dissolved into 25 mL distilled water by adding 5 mL of glacial acetic acid with stirring at 400 rpm using a magnetic stirrer for 12 h in order to form a homogeneous mixture. Alginate hydrosol was prepared by dissolving 1 g of alginate in 25 mL of distilled water by stirring at 400 rpm and allowing it to dissolve overnight (12 h). The formed mixed polymers were the hydrosol solution. Homogenizer (IKA, T18, Ultra Turrax, Staufen, Germany) was used to mix both polymers (2%) for 90 s. Then, the hydrosol was decanted into a polypropylene beaker to make a gel membrane and was chilled for further use. IR absorption of the prepared alginate-chitosan hydrogel was measured using the KBr pellet method at a compression pressure of 2500 lb/m^2 with a FT-IR spectrophotometer (FT-IR 1600 Perkin Elmer Co Japan). Scanning electron microscopy (SEM) studies were carried out on the alginate-chitosan hydrogel after coating with gold-palladium on a SEM (model Joel LV 5600 USA). The methods of measuring the crystallinity of the alginate-chitosan membrane used X-ray diffraction (Diffractometer X-Ray Rigaku Co Japan).

2.3 Immobilization Procedure

The mixtures of alginate-chitosan hydrosol were used as the solid support for the urease and BTB immobilization. Before the hydrosol mixture (50 mL alginate-chitosan) was used, the mixture was added with 2 mL of 32% HCl and then adjusted with NaOH 10% (w/v) to reach pH at 5.28 approximately. Stirring was conducted for ±4 hours at room temperature until the mixture was homogeneous. Afterward, 3 μL of hydrosol mixture was taken and added to 1 μL phosphate buffer (pH 6.5). The buffered mixture was added with 3 μL of urease (enzyme solution in phosphate buffer, pH of 6.5) and 3 μL of BTB (1.5 mg/mL in ethanol). Immediately, 10 μL of the hydrosol mixture was put in a specially designed circular mold (10 mm in i.d. and 1 mm depth). The mixture was stored for 5 days in a refrigerator (4 °C) until a membrane was formed. The final membrane was removed from the mold and stored in a sealed container and kept in a refrigerator (4 °C) for aging until use. Here, the addition of phosphate buffer in the hydrosol process is necessary in order to preserve the enzyme activity in the modified hydrosol matrix.

2.4 Enzyme Activity

Monitoring of the enzyme activity was carried out by determining the formation of ammonia as a result of enzymatic reactions using a reflactance spectrophotometer (by using an optical fiber spectroscopy, USB 2000 spectrometer, Ocean Optic, USA). The enzyme activity is proportional to the product produced. Determination of enzyme activity (5 min) was carried out at pH 7 with a fixed urease concentration and varying urea concentration (5-70 mM).

3 Results and Discussion

3.1 PEC of the Alginate-Chitosan Membrane

Various mass ratios of Na-alginate and chitosan were used (0.5:1; 1:1 and 1:0.5) and it was found that the optimum ratio to produce a good and stable PEC membrane was 1:1. This indicates that at this ratio, the ionic interaction between the NH_3^+ group of chitosan and the COO^- group of alginate form a stronger interaction compared to the other ratios. In addition, the drying temperature and pH of the mixture also greatly affect these ionic interactions. The drying temperature of the alginate-chitosan PEC membrane was optimum at room temperature, even though the aging time required to create a PEC hydrogel membrane was longer (±72 hours). Since it was treated at room temperature, it produced a strong and well-formed PEC membrane. Meanwhile, at a higher drying temperature (60 °C) good formation of the alginate-chitosan membrane was not produced. At 60 °C, weaker ionic bonds can be formed, since the PEC membrane is brittle and easily breaks down. In terms of pH, formation of the alginate-chitosan PEC membrane occurred at a pH of 5.28 approximately. This is because the carboxylate groups of the alginate are present in carboxylate ion form, whereas the NH_2 group of chitosan is protonated. The electrostatic interaction of the two opposing charges of these functional groups causes salt formation, resulting in a well-formed PEC membrane.

The IR spectrum of the alginate-chitosan membrane is shown in Figure 1. There is absorption at the wavenumbers (cm^{-1}): 3429.2 (OH from alginate / $-NH_2$ of chitosan), 2923.9 (CH sp^3), 1577.7 (COO^-). Interaction of carboxylic acid and amine groups may give absorption at the wavenumbers (cm^{-1}): 1740-1630 (C = O) and 1630-1510 (NC = O). However, in fact, there is no C = O and NC = O absorption in the IR spectrum of the alginate-chitosan PEC membrane. This indicates that formation of the alginate-chitosan membrane involves ionic interaction only. A peak at 1398 cm^{-1} indicates stronger electrostatic interaction of the PEC membrane with an alginate and chitosan ratio of 1:1 in mass. The appearance of the mentioned absorption band in the formed PEC membrane

indicates electrostatic interaction between the carboxylic group of the alginate and the protonated amine of the chitosan.

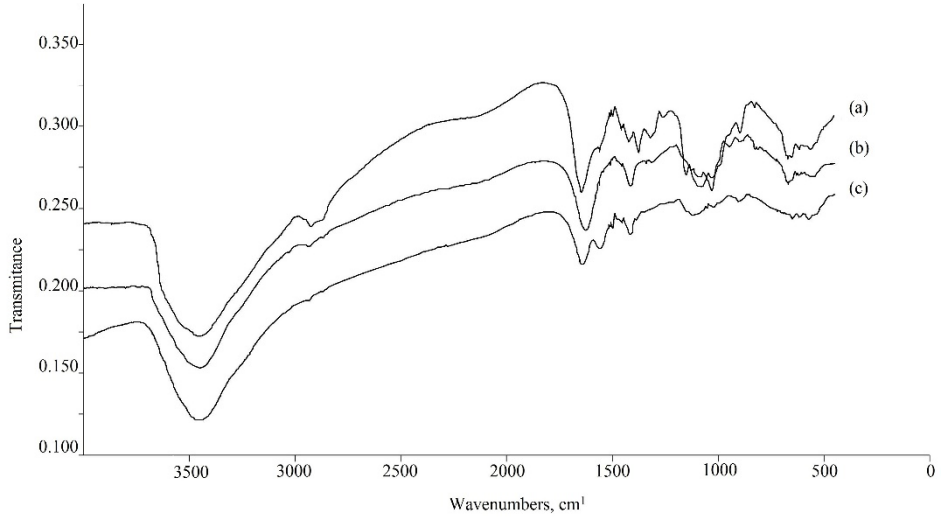

Figure 1 Absorption bands of: (a) chitosan; (b) alginate; (c) alginate-chitosan membrane.

X-ray diffraction pattern was used to assess the interaction between the alginate and the chitosan. The results are shown in Figure 2. The XRD alginate diffractogram pattern shows the characteristics of a natural semi crystal with a diffraction peak at 2θ = 13.61°, which is a characteristic peak of alginate [12]. This show the cristallinity level of alginate with the distance between the crystal lattice is 6.501 based on the Bragg equation [13].

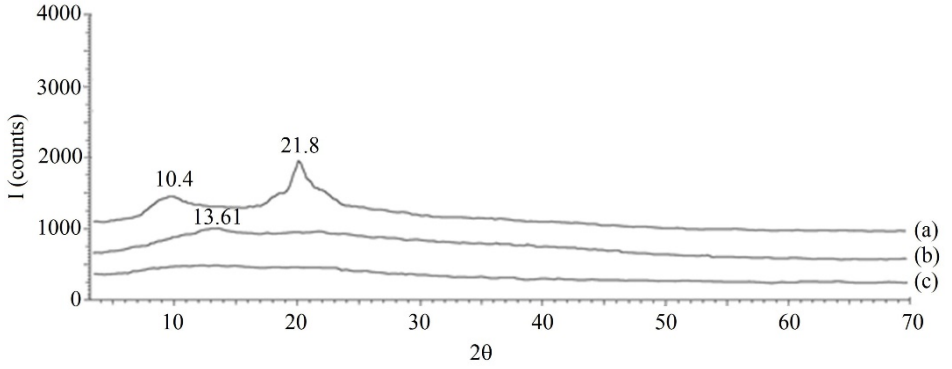

Figure 2 X-ray diffraction patterns of the membrane: (a) chitosan, (b) alginate, and (c) PEC alginate-chitosan.

The XRD chitosan diffractogram pattern showed characteristic diffraction peaks at 2θ = 10.4° and 21.8° [14] with the distance between the crystal lattice at 8.470 and 4.075 [13]. The diffractogram of alginate-chitosan showed a wide peak, indicating the amorphous character of the PEC membrane. When complexing with alginate, the peak of 2θ chitosan became wider due to the breakdown of hydrogen bonds between amino groups and hydroxyl groups in the chitosan [15].

This result is related to electrostatic interactions between the alginate and the chitosan. The XRD pattern showed that alginate-chitosan is formed in amorphous form, which corresponds to a previous report that alginate-chitosan in a composite layer prepared through electrodeposition is also in amorphous form [16]. The alginate-chitosan XRD is characteristic of an amorphous polymer consisting of a structure of dense tissues interpenetrating a polymer chain, cross-linked to one another and implying greater irregularities in the smoothing of chains in the polymers [13].

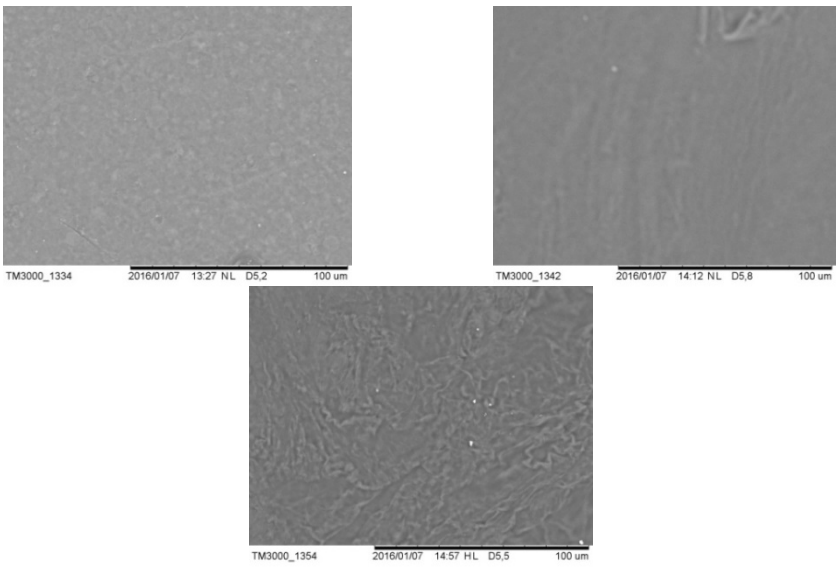

Figure 3 SEM images of surface morphology of: (a) alginate; (b) chitosan; and (c) alginate-chitosan PEC membrane.

The surface and cross-section morphology of the alginate, chitosan, and alginate-chitosan membrane were studied by SEM, as shown in Figure 3. Here, the morphology of the blended alginate-chitosan membrane was less homogenous than the alginate or the chitosan only. It was observed that the alginate-chitosan PEC membrane surface exhibited a fibrous structure with a rough morphology and an irregular form, which is a network of electrostatic

interactions of alginate and chitosan. The difference in surface morphology of the resulted membrane with a single membrane proves that a PEC membrane has been formed. A similar surface morphology of an alginate-chitosan membrane has been reported previously in [9].

3.2 Polyelectrolyte Complex Scheme

Chitosan is a weak base polymer with intrinsic pKa 6.5, whereas alginate consists of carboxyl groups that can be ionized at pH above pKa 4.7 [17]. Therefore at pH 5.28 the amino group of chitosan is protonated while the alginate is in negative carboxylic form. When the two hydrosols are mixed with a ratio of 1:1 there will be electrostatic interaction between the carboxylate groups of the alginate and the protonated amine groups of the chitosan, as shown in Figure 4. Electrostatic interaction is the main interaction in the formation of an alginate-chitosan PEC membrane.

The formation of an alginate-chitosan PEC membrane can be divided into three main steps, including the formation of primary complexes followed by intracomplex formation and ending with intercomplex aggregation, as in Figure 4.

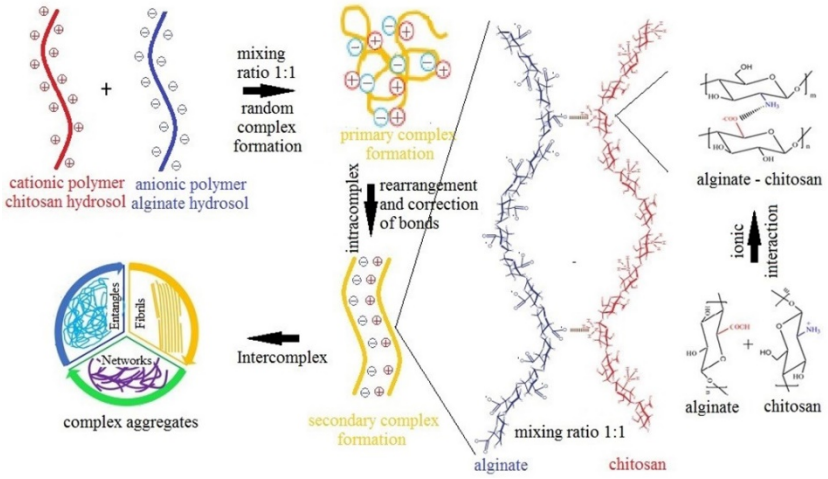

Figure 4 The schematic of possible PEC alginate-chitosan membrane formation.

Mixing alginate and chitosan polyelectrolyte solutions that have opposite charges produces a primary complex that is arranged randomly because the interaction is driven by Coulomb forces and occurs very quickly. The next stage is the formation of a regular secondary complex, occurring due to the repetition or alignment of the polymer chain distortion of the primary complex. After

drying, the PEC membrane undergoes inter-complex aggregation due to hydrophobic or drying interactions, which then produce tissues [18]. The drying time affects the properties of the PEC membrane produced in addition to the pH and ratio factors of the chitosan and the alginate. Adequate drying time (±72 hours) allows the alginate to diffuse deeper into the chitosan, forming a network with suitable pores to immobilize the urease enzyme.

3.3 Enzyme Activity

The successfulness of urease immobilization in the alginate-chitosan PEC membrane was indicated by the change in color of the BTB indicator from yellow to green when reacted with urea. The color change of BTB indicates an increase in pH caused by ammonia formation as hydrolysis product of urea catalyzed by immobilized urease. It confirms that urease was trapped in the cavities of the alginate-chitosan PEC membrane.

Enzyme activity is defined as the quantity of enzyme that causes conversion of 1 µmol substrate per minute at 25 °C in optimal measurement conditions. The formation of enzyme-substrate complexes can be detected directly using a physico-chemical method, namely through changes in the absorption spectrum of the enzyme, which is characteristic when the substrate is added. Figure 5 shows that during the addition of the initial substrate, the reaction rate rises gradually, proportional to the substrate's concentration, so that the rate of reaction that occurs in this condition is a first-level reaction (first-order reaction).

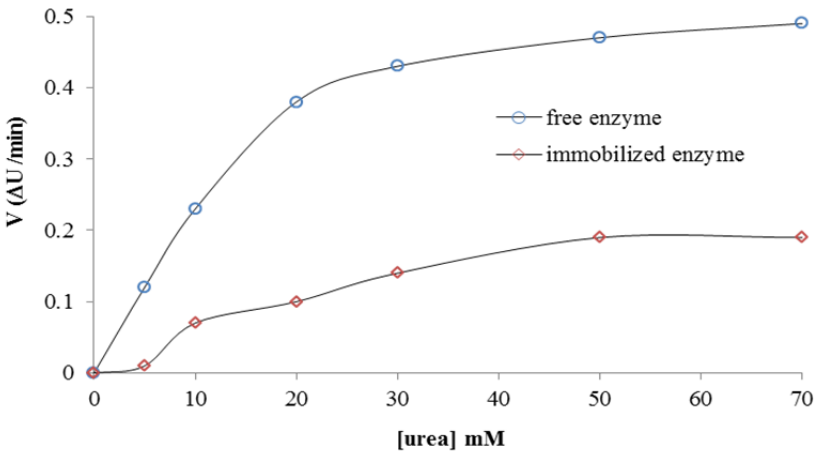

Figure 5 The substrate concentration effect on enzyme activity in free and immobilized conditions.

Upon the addition of substrate to excess it can be seen that the rate is constant until there is no additional reaction rate (the maximum rate is reached). In this situation all enzymes are in complex form with the substrate so that the reaction becomes independent of the substrate concentration, and the said rate of the reaction changes to a zero-level reaction (zero-order reaction). In this case the rate of the initial reaction is directly proportional to the concentration of the enzyme, so the rate-limiting factor in this condition is the concentration of the enzyme.

At the same substrate concentration, the speed of free enzyme catalysis was greater than that of the immobilized enzyme so that the enzyme affinity for the urea substrate was higher when the enzyme was free. This was because the substrate needs to diffuse first through the supporting material and then release the product, so that the enzyme response in immobilized state is not as fast as when the enzyme is free. Difficult access to the substrate causes lower activity of the immobilized enzyme than free enzyme activity. A similiar result was found in previous studies [19,20].

Another factor that influences urease activity is the location and orientation of the substrate in relation to the catalytic group and the stress and change in the bonds of the object by the impulse of enzyme placement. The enzyme binds to the substrate molecule in such a way that the bond to be catalyzed not only lies close to the catalytic group, but also points precisely to the cluster, thus significantly increasing the possibility of ES complex entry into the transition. Substrate binding can encourage conformational changes in enzyme molecules, which causes stress on the active side structure and also changes the bound substrate, thus helping to bring the ES complex to a transition state. This change is called the right change in enzymes by the substrate.

All of the above factors will occur effectively when the enzyme is in solution, allowing the enzyme to move freely to adjust to the location and orientation of the substrate, but also the urge for enzyme placement is easier because when enzyme conformation changes, a different space is required than during enzyme relaxation. Therefore, enzyme activity becomes lower when the enzyme is immobilized in a matrix that limits its motion space.

4 Conclusion

The PEC of the alginate-chitosan membrane as supporting material for immobilizing urease was produced by interacting alginate and chitosan hydrosols with a mass ratio of 1: 1 at a pH of 5.28. The FTIR spectra, XRD patterns and SEM assay confirmed that formation of alginate-chitosan membrane PEC successfully took place. The color change of the alginate-

chitosan membrane from yellow to green when reacted with urea showed that urease was loaded successfully in the formed PEC membrane, therefore the alginate-chitosan PEC membrane has good characteristics as a matrix for urease immobilization and has further potential applications, for example as a matrix for biosensing systems.

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