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Chitin Nanowhisker and Chitosan Nanoparticles in Protein Immobilization for Biosensor Applications

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Abstract

In this study, bovine serum albumin (BSA) was used as a model protein for the optimization of protein immobilization on chitin nanowhisker and chitosan nanoparticles under different conditions. Chitosan nanoparticles showed better results than chitin in this immobilization purpose. The immobilization at 15 minutes, pH 6 and 20-25°C was the optimal condition. Subsequently, glucose oxidase (GOD), enzyme used for glucose determination, was applied in order to investigate the optimum immobilization condition on chitosan nanoparticles. The optimal condition prove to…at pH 7, 30°C and 0.5 mg/ml of initial GOD concentration via immobilization at 15 minutes. Chitin nanowhisker and chitosan nanoparticles in this study had 300 and 39 nm in size, respectively. These results will be further used for the development of glucose electrodes.

Key words : chitin; chitosan; nanoparticles; glucose oxidase; glucose biosensor

Introduction

Chitin is a polysaccharide found in the outer skeleton of insects, crabs, shrimps, and lobsters and in the internal structures of other invertebrates. Chitin is composed of ß(1-4) linked units of the amino sugar N-acetyl-glucosamine, and is the main source of production of chitosan, which is used in a number of applications, such as a flocculating agent, a wound healing agent, a sizing and strengthening agent for paper, a delivery vehicle for pharmaceuticals and genes as well as the biomaterial for immobilization, especially of biomolecules. Chitosan is an attractive natural biopolymer with the presence of reactive amino and hydroxyl functional groups.[4] It has shown favorable biocompatibility characteristics.[3, 1, 2] as well as the ability to increase membrane permeability.[7] Moreover, it is one of the most promising immobilization matrices due to an excellent membrane-forming ability, good adhesion, low cost, nontoxicity, high mechanical strength, and hydrophilicity.[8] as well as the improvement of stability. These properties have prompted extensive applications of chitosan as a matrix for enzyme immobilization. All of these excellent properties lead chitosan to be a proper material for enzyme immobilization, especially within biosensor applications that are mostly concerned with working of enzymes for detection mechanisms. Moreover, this excellent material, chitosan, can be prepared in nanoparticle form, which could enhance the benefit of improving characteristics in biosensor works. Many research papers mainly reported the preparation of chitosan in order to immobilize many enzymes for usage in biosensor, especially electrochemical detection.[5, 9] However, there were few articles in regard to the application in direct immobilization with chitosan nanoparticles. This study, therefore, aims at targeting protein immobilization with chitin nanowhisker and chitosan nanoparticles in order to find suitable material for subsequent applications with enzyme glucose oxidase (GOD) so as to fabricate an efficient biosensor to either improve its characteristics or use it as a model for further applications.

Materials and Experimental Procedures

Materials

Chitin and Chitosan with degree of deacetylation (DD) of 0.45 and 0.95, respectively, (M_v = 6.7 × 10^5 Dalton) were purchased from Seafresh Chitosan (Lab) Co., Ltd., Thailand. BSA,

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Glucose oxidase (GOD; from *Aspergillus niger*; EC 1.1.3.4, type X-S; 100000 – 250000 unit g^{-1}), Tripolyphosphate (TPP) and D-glucose were obtained from Sigma. All the other chemicals were of analytical grade and no further purification was required. Deionized water was used for all experimental steps. All buffer solutions were pumped with O_2 gas before usage in the amperometric detection.

Chitin Whisker and Chitosan Nanoparicles Preparation

1 g chitin was hydrolyzed by 100 ml 3 N HCl in refluxing flask at 105°C for 3 hr. It was then centrifuged at 10,000g for 5 mins to separate acid solution; this step has repeated 2 times. After that it was dialyzed with distilled water until a pH of 7 was reached and chitin whisker powder was obtained by lyophilization.

20 mg chitosan were dissolved in 40 ml of 2.0% (v/v) acetic acid. Then 20 ml of 0.75 mg/ml TPP was dropped into a beaker at room temperature. After that chitosan solution was sonicated for 5 mins and centrifuged for 15 mins at 6000g in order to obtain chitosan nanoparticles. These chitosan nanoparticles could be stably stored in distilled water; however, sonication for 10 seconds should be applied before a further immobilization step.

The morphological characterization of the chitin whisker and chitosan nanoparticles were evaluated by a Transmission electron microscopy and Nanosizer.

Protein Immobilization

Both chitin whisker and chitosan nanoparticles were resuspended in deionized water and sonicated for 10 s before immobilization. The dilution of 1:100 was made afterwards with difference buffers at various pH and temperatures. For the immobilization study, chitin whisker or chitosan nanoparticles were used mixed with BSA or GOD at ratio 1:1. The immobilization times were 1 min and 15 mins of protein and chitin or chitosan incubation. Centrifugation was used to receive the supernatant after finishing immobilization to determine the concentration of remaining BSA or GOD by UV absorption at 280 and 277 nm, respectively.

Electrochemical Measurements

GOD-chitosan electrode was produced by mixing GOD-chitosan nanoparticles with carbon paste and used as a working electrode. Ag/AgCl and platinum wire were used as reference and counter electrodes, respectively. The potential of +700 mV vs Ag/AgCl in 0.1 M sodium phosphate buffer (PBS) pH 7.0 was applied to the system at room temperature. During measurements, rotation of the working electrode was constant.

Results and Discussion

Morphology and Characteristics of Chitin Nanowhisker and Chitosan Nanoparticles

The chitin nanowhisker and chitosan nanoparticles were evaluated by the means of TEM as shown in Figure 1. The size of chitin nanowhisker and chitosan nanoparticles, which is based on the method by hydrolyzation and ionic gelation, was minimum at ~ 300 and 39 nm, respectively. However, the aggregation of particles was observed. It might be solved by using the addition of some surfactants in order to avoid the effects of charge and size of these particles. Moreover, the results from Nanosizer (Malvern instrument) showed the average diameter of chitin nanowhisker and chitosan nanoparticles for 420 and 215 nm respectively Table 1. The results differed from observation via TEM because of the difference in the principle technique of each measurement. Nanosizer is based on the average size of particles in the sample, causing the detection of particles' groups. The results from Nanosizer are normally bigger than those from TEM.

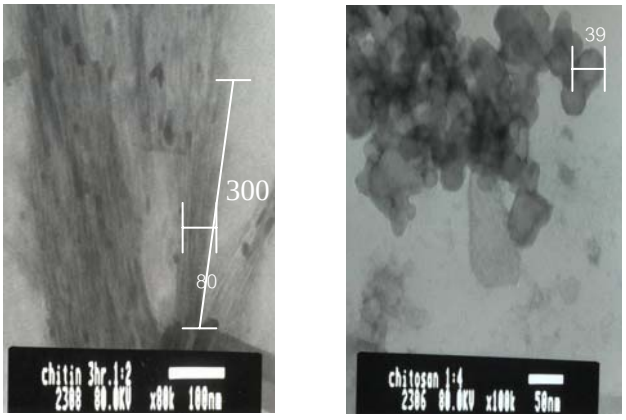


Figure 1. TEM images of the chitin nanowhisker (left) and chitosan nanoparticles (right)

Table 1. Mean diameter of chitin and chitosan under different conditions by using Nanosizer (Malvern instrument)

Condition	Size (nm)
Raw chitin	$110x10^3$
3 hr digested chitin	420
Raw chitosan	1000
1 min sonicate chitosan	438
5 mins sonicate chitosan	215

Optimal Condition for Protein Immobilization

The optimal pH for BSA immobilization with chitin nanowhisker and chitosan nanoparticle is shown in Figure 2(A). The time of immobilization was 15 min at 25°C (data is not shown). If making a comparison between chitin nanowhisker and chitosan nanoparticle, the suitable material for protein immobilization is chitosan nanoparticle at pH 6 (acetate buffer) because it provided the least remaining BSA. Therefore, we chose chitosan nanoparticle for further GOD immobilization in order to fabricate glucose sensor.

The effect of pH on the GOD-chitosan nanoparticle immobilization is shown in Figure 2(B). The optimal pH was 7.0 and the optimal temperature 30°C (data are not shown). The pH value is one of the most influential parameters altering enzyme activity in an aqueous medium. Immobilization is likely to result in a conformational change of the enzyme, which leads to inactivity of the enzyme.[6] as well as appearance charge of enzyme in order to give interaction with chitosan nanoparticles high strength. Enzyme glucose oxidase has pI at 4.2. It revealed the negative charge in this case, which is perfectly fit with chitosan nanoparticles. The initial concentration of GOD was varied in order to obtain the highest amounts of GOD after immobilization. The suitable initial GOD concentration was around 0.5 mM. It provided higher retained GOD amounts in GOD-chitosan nanoparticle whereas the increasing GOD concentration did not improve the immobilizing ability as shown in Figure 3(A). The saturation of GOD was at 1 mg/ml after immobilization.

In addition the storage stability was investigated as illustrated in Figure 3(B). The GOD-chitosan nanoparticle depicted better long time storage. The recovery activity of GOD-chitosan nanoparticle was still higher than 90% after 21 days. In contrast, the recovery activity of the free enzyme was only 30% after 9 days. The results indicated that the GOD-chitosan nanoparticle had good storage stability.

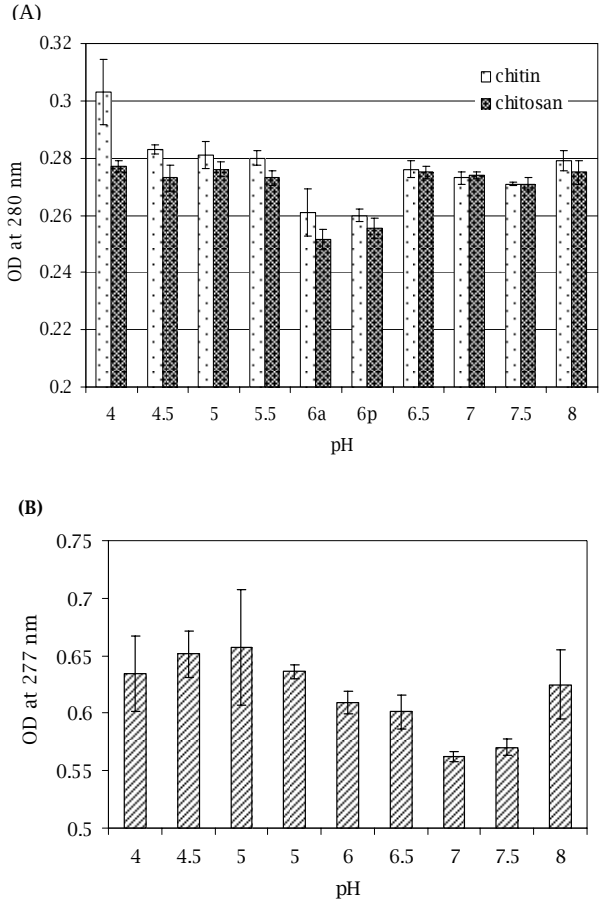
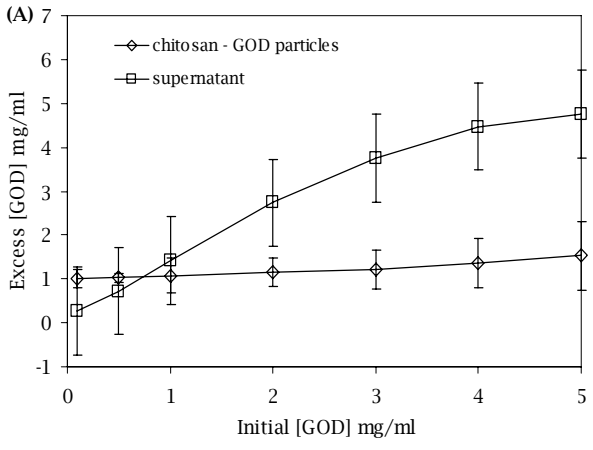


Figure 2. (A) The amount of BSA (represented as OD at 280 nm) remaining in chitin nanowhisker and chitosan nanoparticle solution after immobilization, by variation of pH with acetate buffer (a) and phosphate buffer (b); (B) the amount of GOD (represented as OD at 277 nm) which remains from chitosan nanoparticle solution, by variation of pH.



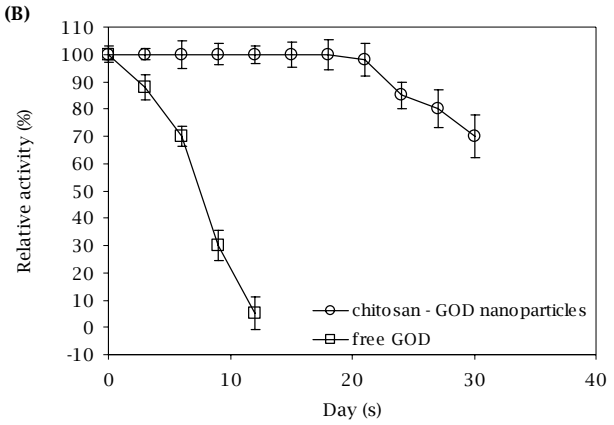


Figure 3. (A) The effect of initial GOD concentration to the optimal immobilization ; (B) storage stability of the free and immobilized enzymes that were stored at 4 °C for a long time.

Performance GOD-chitosan electrode

The amperometric responses of GOD-chitosan electrode with the additions of various glucose concentrations (0.5, 1.0, 2.0 and 5.0 mM) in 0.1M PBS (pH 7) at an applied potential of +700mV (vs. Ag/AgCl), room temperature is shown in Figure 4. The electrode exhibits a rapid and sensitive current response for the changes of glucose concentration and indicates the excellent electrocatalytic behavior of the electrode.

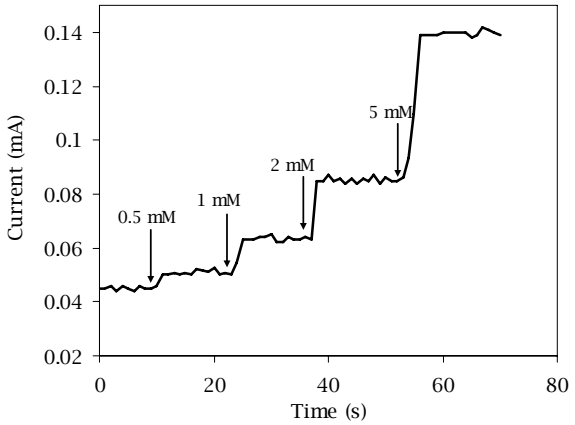


Figure 4. Amperometric responses of GOD-chitosan electrode with the additions of 0.5, 1.0, 2.0 and 5.0 mM glucose in 0.1M PBS (pH 7) at applied potential of +700mV (vs. Ag/AgCl), room temperature.

Conclusions

Chitin and chitosan are biological materials which are good for protein immobilization. In this study, the chitosan nanoparticles showed better BSA immobilization characteristics than chitin whisker. Their morphology and some characteristics depicted the good size of nanoparticles and suitable charge for target protein immobilization. However, in order to study the protein immobilization, we also have to determine the amount of protein per particle which could be included in a further study.

In the biosensor application of chitosan nanoparticles, we developed a glucose oxidase – chitosan (GOD-chitosan) electrode for the detection of glucose. The signals of glucose detection by amperometric method were good. However, there should be further study of other characteristics of this glucose sensor and the effect of interference as well as real samples detection. Nevertheless, these features provide scope for utilizing the methodology proposed in the present study to immobilize other biomolecules in the process of fabricating novel biosensors by using waste and cheap materials which are obtained from other industries.

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