J Membrane Biol (2014) 247:549–559
DOI 10.1007/s00232-014-9671-y

Development of Silica Gel-Supported Modified Macroporous Chitosan Membranes for Enzyme Immobilization and Their Characterization Analyses

Wen-Yi Yang · Munusamy Thirumavalavan · Madasamy Malini · Gurusamy Annadurai · Jiunn-Fwu Lee

Received: 15 January 2014 / Accepted: 29 April 2014 / Published online: 21 May 2014

Abstract The present work was aimed at developing stability enhanced silica gel-supported macroporous chitosan membrane for immobilization of enzymes. The membrane was surface modified using various cross-linking agents for covalent immobilization of enzyme *Bovine serum albumin*. The results of FT-IR, UV–vis, and SEM analyses revealed the effect of cross-linking agents and confirmed the formation of modified membranes. The presence of silica gel as a support could provide a large surface area, and therefore, the enzyme could be immobilized only on the surface, and thus minimized the diffusion limitation problem. The resultant enzyme immobilized membranes were also characterized based on their activity retention, immobilization efficiency, and stability aspects. The immobilization process increased the activity of immobilized enzyme even higher than that of total (actual) activity of native enzyme. Thus, the obtained macroporous chitosan membranes in this study could act as a versatile host for various guest molecules.

Keywords Macroporous membranes · Chitosan · Silica · Cross-linking · Enzyme · Immobilization

Introduction

The rapid development in biotechnology is fueling the demand for reliable, efficient separation and purification methods for biologically active species such as enzymes. These enzymes can be adsorbed or immobilized especially onto matrices bearing functional groups (Shentu et al. 2005). In general, the adsorption techniques resulted into weak bonding and the degree of stabilization will be very low (Bautista et al. 1999). Thus, the immobilization technique (Nisnevitch and Firer 2001) has become an important goal as it can offer several advantages including reuse, ease in separation, and improvement in stability. Such immobilization fixes the enzymes or proteins onto solid inert support either by covalent bonding, physical adsorption, or encapsulation (Bautista et al. 1999). The selective and specific binding of the enzymes is based on electrostatic, ion exchange, hydrophobic, or affinity interaction between the support surface and enzyme (Kawai et al. 2003). One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme and the extent of such stabilization depends on the enzyme structure, the immobilization methods and the type of support. Thus, preparing a suitable candidate for enzyme immobilization has become a thrust area of research. The affinity column chromatography was found to be effective for enzyme separation, but, however, with limitations (Suen et al. 2000). Recently, the affinity membrane has emerged as an alternative to affinity column chromatography overcoming the limitations (Zeng and Ruckenstein 1996a, b, 1998). Commercially available microporous membranes,

Electronic supplementary material The online version of this article (doi:10.1007/s00232-014-9671-y) contains supplementary material, which is available to authorized users.

W.-Y. Yang · M. Thirumavalavan · J.-F. Lee (✉)
Graduate Institute of Environmental Engineering, National Central University, Chung-Li, Taoyuan County 320, Taiwan
e-mail: jflee@ncuen.ncu.edu.tw

M. Malini · G. Annadurai
Environmental Nanotechnology Division, Sri Paramakalyani Centre for Envrionmental Sciences, Manonmanium Sundaranar University, Alwarkurichi 627412, Tamilnadu, India

as well as synthetic membranes containing functional groups have been employed for this purpose. However, these membranes are (Zeng and Ruckenstein 1996a, b, 1998) hydrophobic, quite inert and they can not easily acquire macroporous structure. Hence they required respective chemical modifications before they can be used. Generally, the affinity membranes are either operated as macroporous membranes or fiber membranes (Singh and Ray 1998; Okuyama et al. 1997). In recent times, novel macroporous chitosan membranes have been developed and they acquire controlled pore size, good mechanical strength, chemical stability, as well as biocompatibility (Beppu and Santana 2001). In addition to this, chitosan contains a large number of reactive hydroxyl (OH) and amine (NH_2) groups due to which chitosan also possess enhanced hydrophilicity. Consequently, it is no longer necessary to increase the number of these groups of the chitosan as they have already possessed significant amount of reactive groups (Zeng and Ruckenstein 1996a, b, 1998). These reactive groups can be readily modified using different ligands and also easily coupled with enzymes (Singh and Ray 1998) and depending upon the different interactions between ligands and ligates, various type of macroporous chitin/chitosan membranes can be developed for the purification of proteins and enzymes. Thus, chitosan can be an outstanding candidate for affinity membranes (Zeng and Ruckenstein 1996a, b, 1998). However, the macroporous chitosan membrane obtained in earlier report (Zeng and Ruckenstein 1996a, b, 1998) did not acquire significant or satisfactory mechanical strength. Hence, there is a need of new kind of porogen or new pathway to obtain macroporous chitosan membranes that possess suitable mechanical properties. Thus, silica particles were found as a suitable porogen as in contrast to chitosan these silica particles are insoluble in acidic solutions but at the same time soluble in alkaline solutions. These converse properties allow us to control the size of the pores during the process in the preparation of chitosan membranes. Since in many cases, the biomolecules are eluted in acidic conditions, the chitosan membrane must be cross-linked to prevent its dissolution. These cross-linked chitosan membranes are insoluble both in acidic and basic solutions. So far only limited studies were carried out to develop such macroporous chitsan membranes, and the detailed studies were also not performed. Thus, the major aim is to prepare a suitable macroporous chirosan membrane and in this present work, we have reported the preparation and the application of silica-supported cross-linked macroporous chitisan membranes using different cross-linking agents (1,4-butanedioldiglycidyl ether, glutaraldehyde, epichlorohydrin). The effect of concentration of cross-linking agents was mainly studied. Immobilization of enzyme *Bovine serum albumin* (BSA) onto cross-linked macroporous chitosan membranes was then carried out. Various parameters on immobilization study such as effect of pH on the immobilization efficacy, effect of initial enzyme concentration, activity assays of free and adsorbed enzyme, and dependence of enzyme activity on pH and temperature were extensively studied. These membranes possess affinity ligand (one N-acetyl-D-glucosamine group for each polymer unit), and hence they can be successfully employed for separation or purification of enzymes and proteins. Also, since they contain chemically reactive groups they can be easily coupled with various affinity ligands. Despite chitosan contains high content of amine and hydroxyl functional groups, the amine groups are sole responsible for the crystallinity and diffusion properties of the chitosan (Okuyama et al. 1997). Due to this responsibility of amine group, these chitosan membranes can serve as a weak anion exchanger. Hence, these cross-linked macroporous chitosan membranes are much cheaper and have a high adsorption capacity for negatively charged biomolecules. Further, these can be even employed to separate various binary mixtures also. Thus, these macroporous membranes may also be useful when they are operated in an ion exchange mode and these membranes upon different modification processes will be useful for various purposes depending upon the requirement of applications.

Thus, in this study for immobilization of enzymes, silica gel-supported cross-linked macroporous chitosan membrane was prepared by phase inversion method. This silica gel in the core of the membrane could act as a rigid support and the chitosan layer could provide a sufficient amount of amino groups for enzyme coupling. Also enzymes could be immobilized only on the surface and hence the loss in the activity and diffusion limitation problems will be reduced, and the presence of macropores would also facilitate the incorporation of some other required materials for even different kinds of purposes.

Materials and Methods

Chemicals

Deionized distilled water was used to prepare all solutions. Chitosan (medium molecular weight, $M_w \sim 750{,}000$) was obtained from Sigma. Silica gels (sizes in the range 15–40 μm) were obtained from Silicycle. Cross-linking agent, 1,4-butanedioldiglycidyl ether (95 %) was obtained from Aldrich, glutaraldehyde solution (25 wt% in H_2O) was obtained from Sigma, and epichlorohydrin (99 %) was obtained from Alfa Aesar. Acetic acid and acetic anhydride were obtained from TEDIA. Sodium hydroxide and glycerol were supplied by J. T. Baker. Enzyme BSA was

purchased from Sigma. All other chemicals and reagents used in this study were of analytical grade and used without any further purification.

Preparation of Chitosan Macroporous Membrane

The chitosan macroporous membranes were prepared as follows: a solution of chitosan was first obtained by dissolving 1 g of chitosan in 100 mL of 1 vol% aqueous acetic acid solution. To this solution, silica particles (weight ratio of silica to chitosan of 15:1) were added, followed by vigorous stirring for 6 h in order to disperse them uniformly. Then, the solution was poured onto a rimmed glass plate and the liquid was allowed to evaporate. The dried membrane was immersed in a 5 wt% aqueous NaOH solution and kept at 80 °C for 2 h in order to dissolve the silica particles and to generate a porous membrane. Finally, the porous membrane was washed with distilled water to remove the remaining NaOH. In order to prevent its shrinkage during drying, the membrane was immersed in a 20 vol% aqueous glycerol solution (softening agent) for 30 min and, after the excess glycerol solution was removed, placed on a glass plate and allowed to dry. Thus, a soluble but strong and flexible mechanically stable macroporous chitosan membrane (CM15) without shrinkage was obtained.

The Cross-Linking of Macroporous Chitosan Membrane

The chitosan membrane was cross-linked by various cross-linking agents such as 1,4-butanedioldiglycidyl ether, glutaraldehyde, and epichlorohydrin solutions of various concentrations (1, 2, 3, and 4 %) at room temperature for 24 h. The cross-linking was carried out at mild alkaline condition (NaOH) (Wei et al. 1992) in the presence of small amount of sodium borohydride ($NaBH_4$) to avoid any oxidation. Thereafter, the membranes were washed with distilled water until reaching neutral pH to remove excess of cross-linking agents and subsequently dried in air. The structural formulae of cross-linking agents are shown in Fig. 1a.

Immobilization of Enzyme BSA onto Cross-linked Macroporous Chitosan Membranes

The immobilization of enzyme (BSA) was carried out at room temperature by thoroughly mixing macroporous chitosan membranes (as-synthesized and cross-linked) and enzyme BSA for 3 h in 0.05 M citrate buffer (pH 5.6) and the initial immobilization concentration was adjusted (0.5–1.0 mg/mL). Finally, the immobilized membranes were collected by centrifugation, and the resulting filtrate was separated to obtain the activity of supernatant BSA.

Characterization Techniques

The flow rate and the average pore diameter (pore size) were measured by capillary flow analysis using capillary flow meter PSM 165. SEM was carried out on an HITACHI-S-800, field emission scanning electron microscope. TEM was carried out on a transmission electron microscopy (JEM-2010 type). A Neclit 6700 model, Fourier transform infrared spectrometer (FT-IR) was used to record the FT-IR spectra. Thermogravimetric analyses (TGA) were performed with Universal V4.4A TA Instruments. UV–visible spectra were obtained using UV–visible spectrophotometer (Hitachi U-2800 A).

Results and Discussions

Significance of Preparation Conditions of Macroporous Chitosan Membrane

This study was mainly focused on the preparation of macroporous chitosan membrane by casting method which involves various important steps. First, the solution containing chitosan and porogen (silica) was partially evaporated. Then, the pores were generated on membranes by adding the solvent (NaOH) for porogen, but at the same time the chitosan was not dissolved by the same. Generally chitosan dissolves in acidic solution, but not in alkaline solution. Finally, the process of heat treatment was applied in order to improve the porosity and stability of the

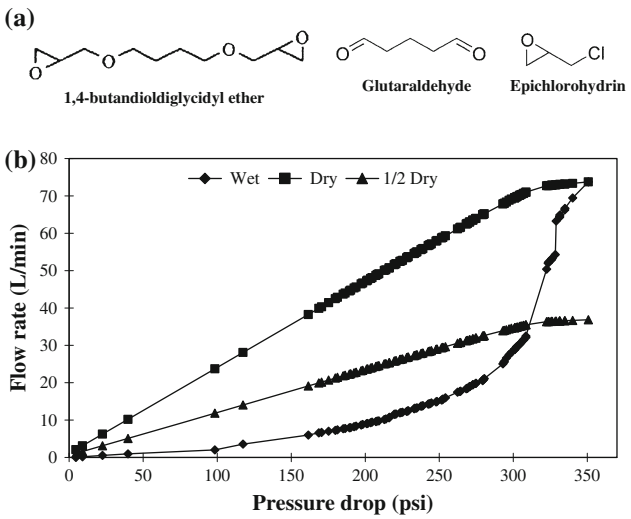

Fig. 1 **a** Structural formulae of cross-linking agents; **b** Flow rate through CM15

membranes. In order to meet the required efficiency, the macroporous chitosan membrane was modified using three different types of cross-linking agents before enzymes immobilization. Thus, the variation in the characteristics of membranes is mainly determined through porosity control and functional activity by cross-linking. The obtained membranes were then characterized using different techniques in order to understand their different physicochemical and textural properties.

Flow Rate Analysis and Average Pore Size

The flow rate through different macroporous chitosan membranes under different conditions is shown in Fig. 1b. The results showed that the flow rate is very sensitive to drying conditions, and the flow rate increased if the membranes were dried well. The flow rate through dried membranes is greater than that of wet membranes. The flow rate through the half dried membrane is in between the dried and wet membranes. Due to the compression at the high pressure, the flow rate curve on pressure drop is nonlinear (Zeng and Ruckenstein 1996a, b, 1998). The particle diameter and the porosity are important factors for the support materials (Oh and Kim 2000). With larger macrospheres, there are relatively larger pores into which enzymes can be easily immobilized on the surface. The average pore size (pore diameter) of the chitosan membrane CM15 prepared with weight ratio of silica to chitosan of 15:1 is 0.0212 μm.

Modification of Macroporous Chitosan Membrane

Usually, the biomolecules are often eluted at acidic pHs during separation process. However, at acidic pH, the macroporous chitosan membranes are less stable and soluble in acidic solutions. Hence, to avoid such dissolution the cross-linking of chitosan is required to maintain the stability of chitosan membranes. Also such cross-linking can increase the enzymatic stability even on immobilization. At the same time, the cross-linking of chitosan diminishes the ligand density also due to the reaction of cross-linking agents with the functional groups of chitosan. As discussed earlier, it is well known that among two functional groups (OH and NH_2) present in chitosan, amino group (NH_2) is more active than OH group, and thus it is advised to maintain the density of amino group in carefully selecting the cross-linking agents which are capable of reacting with only OH group and not with amino group. Thus, in this study we have chosen the three selective cross-linking agents as described in experimental section. The cross-linking was also carried out using different concentrations of cross-linking agents. Thus, the cross-linked macroporous chitosan membranes have numerous advantages such as; (i) the reactive membrane can be prepared without any activation steps; (ii) it can be easily used for enzyme immobilization under mild experimental conditions; (iii) the desired amount of epoxy and amino groups can be created on the surface; and (iv) the reusability of the membrane support may provide economic advantages for large scale application. Thus, it is presumed that among the cross-linking agents selected in this study, 1,4-butanedioldiglycidyl ether is considered to be efficient as it posses increased epoxy group as well increased oxygen content. These cross-linking agents can block the amino groups and make chitosan structure more inert and resistant to acidic media. At acidic and neutral conditions, a nucleophilic attack by the amino groups of macroporous chitosan membrane on the olefinic carbon atom occurs and the intermediate compounds could further associate to form cross-linked networks. At basic condition, nucleophilic attack by OH groups occurs via aldol condensation to form intermediate aldehyde groups. It is implied that these cross-linking agents would be polymerized prior to cross-linking (Wang et al. 1998), and thus the cross-linked macroporous chitosan membrane networks prepared at strong base consist of primary polymer chains of macroporous chitosan membrane and long cross-link bridges of cross-linking agents. Hence, cross-linking agents 1,4-butanedioldiglycidyl ether and epichlorohydrin could affect only OH functional groups present in the chitosan molecule. But however, the cross-linking agent glutaraldehyde could possibly affect amino (NH_2) group rather than OH group resulting into Schiff's base condensation (Uragami et al. 1994). Cross-linking with glutaraldehyde showed to produce more hydrophobic structures in chitosan membranes, which then interfere in the interaction with water and ions and changes their mechanic characteristics (Beppu et al. 2007). Thus, even though the aim of this work was to protect the amino content of chitosan, glutaraldehyde has preference to attack amino group rather than hydroxyl group. In the absence of any amino group, then it may interact with hydroxyl group. Thus, the glutaraldehyde cross-linked macroporous chitosan membrane may differ from other cross-linked membranes in this work. Actually, enzyme immobilization may not occur on cross-linked units in the case of glutaraldehyde cross-linked macroporous chitosan membrane, but still the cross-linking of glutaraldehye can enhance the stability of macroporous chitosan membranes in over all and thus facilitate the enzyme immobilization. Thus, our experimental results indicated that after cross-linking, the stability of membranes was improved (Ge et al. 2000). Since, the prepared macroporous chitosan membranes posses silica gel, these membranes have received great attention (Asaeda et al. 2005; Kuraoka et al. 2000) due to its immobilizing ability toward various organic functional groups. Thus, different

enzymes were immobilized onto these membranes to achieve high selectivity of enzymatic reactions with the increased chemical and mechanical properties of the support (Bautista et al. 1999). In this respect, the presence of silica gel provided a wide range of advantages such as no change in swelling and porosity with pH, the enzymes are not subjected to microbial attack and high volume productivity. These support systems could provide a large surface area, and therefore, the enzyme could be immobilized only on the surface and would also provide less diffusion limitation problem than that of the porous supports (Oh and Kim 2000). The presence of silica gel could enhance the immobilization process as it combines the selectivity of enzymatic reactions with its chemical and mechanical properties. Also, the silica gel would definitely reduce the risk usually arises when trying to obtain the enzyme immobilization onto the surface of the support which is limited by a reduced available surface area, because it has an ability of immobilizing organic functional groups onto its surface using some silane coupling agent.

TGA, FT-IR, SEM and UV–Vis Analyses

The presence of reactive functional groups on macroporous chitosam membrane was identified using FT-IR technique. The effect of cross-linking agents was also effectively confirmed by FT-IR analysis. Fig. S1a (See Fig. S1) shows the FT-IR spectra of macroporous chitosan membranes before and after cross-linking. The peak around 3,480 cm^{-1} is due to ν(O–H) and ν(NH_2) (Zakaria et al. 2012). The peak at 2,900 cm^{-1} is due to alkyl ν(C–H). The peak around 1,600 cm^{-1} is due to ν(N–H), the peak around (1,300–1,500) cm^{-1} is due to deformation from alcoholic and phenolic ν(C–OH) and ν(C–O–C). The peak around (1,050–1,100) cm^{-1} is due to ν(C–O) of chitosan (Anjali Devi et al. 2005; Beppu et al. 2004; Manjubala et al. 2006) and the peak around 800 cm^{-1} is due to ν(Si–O–Si) and aromatic ν(C–H). The effect of concentration of cross-linking agents can be explained based on the observation of changes (increase) in the peak intensity of respective peaks due to ν(C–H), ν(C–O–C), and ν(C–H) at 2,900 cm^{-1}, (1,300–1,500) cm^{-1}, and 800 cm^{-1} ,respectively, as shown in Fig. S1b (See Fig. S1). Thermogravimetric analyses of the membranes were conducted from room temperature (RT) to 800 °C. The TGA and DTA curves are shown in Fig. 2. It can be seen that TG curve is a smooth curve with only one weight loss step and that there is only one peak on the DTG curve, indicating that the thermal degradation of macroporous chitosan membranes is simple and is a one-step reaction (Hong et al. 2007). The weight loss curves of the chitosan membranes in the range 200–400 °C are associated with the decomposition of chitosan (Desai et al. 2008). The SEM of different membranes obtained by

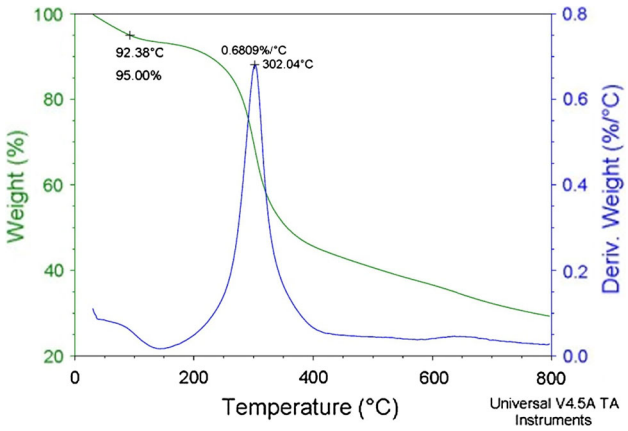

Fig. 2 TGA curve of CM15

various systems is shown in Fig. 3. As seen in Fig. 3, the pores of as-synthesized membrane are distributed uniformly indicating the uniform distribution of silica particles also. The chitosan macrosphere was in the form of spongy hollow fibers. However, the SEM of membrane alone showed more flat surface with less distributed holes comparatively to that of cross-linked one. Also, the comparison of the SEM pictures of chitosan membrane before and after cross-linking indicated that graft branches formed on the pores of the hollow fibers that the network structure of the resin phase swelled (Iwata et al. 1991). Thus, the addition of cross-linking agent blocked the pore entrance of the hollow fibrous membranes, and thus definitely reduced the pore size. Upon enzyme immobilization the membranes still swelled more. Thus, the disparity in the surface morphology as witnessed in Fig. 3, confirmed the successful cross-linking as well effective immobilization of enzyme. The effective cross-linking phenomenon can also be confirmed by analyzing the UV–visible spectra of cross-linked macroporous chitosan membranes as shown in Fig. 4. The UV–visible spectra of cross-linking agents and cross-linked membranes were recorded at various pH values (pH 1.2, 5.0, 7.4, 10.0, and 13.2). At all pH values except pH 13.2, 1,4-butanedioldiglycidyl ether showed one absorption peak at 240 nm, but at pH 13.2, it showed a red shift to 270 nm. However, cross-linked chitosan membranes obtained using 1,4-butanedioldiglycidyl ether showed the random absorption peaks at various pH values (acidic, neutral, and alkaline pH) as shown in Fig. 4. This due to the fact that the nature of nucleophilic attack to form two ring openings may require different rates under different pH values, and hence the intermediate formed may be also different from each other. However, glutaraldehyde showed two absorption peaks at 240 and 265 nm at all pH values and epichlorohydrin showed one absorption peak at 265 nm at all pH values. Also the UV–visible spectra of cross-linked

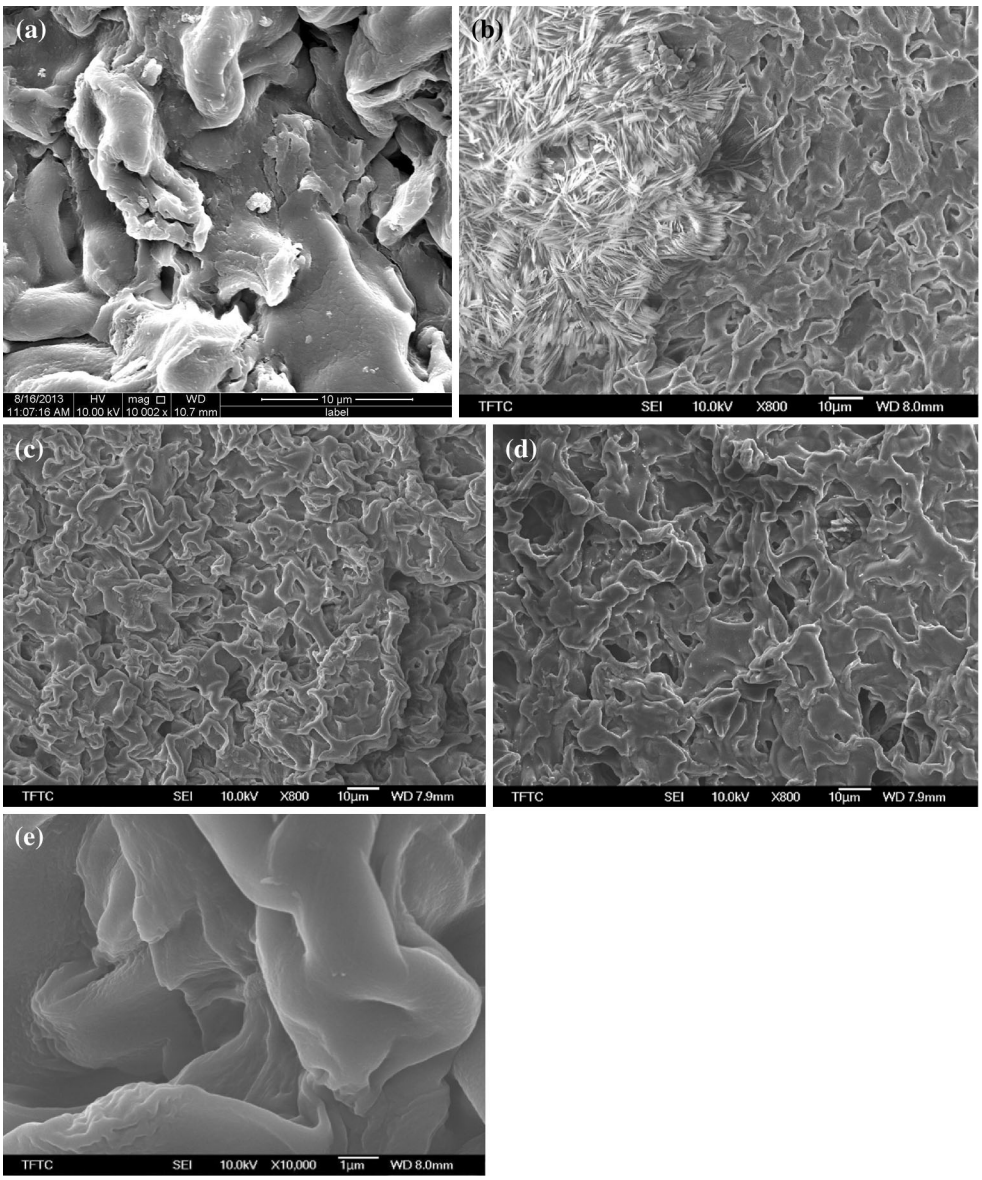

Fig. 3 SEM images of **a** CM15; **b** cross-linked C15 using 1 % 1,4-butanedioldiglycidyl ether; **c** cross-linked C15 using 1 % glutaraldehyde; **d** cross-linked C15 using 1 % epichlorohydrin; and **e** 50 ppm BSA immobilized onto cross-linked C15 using 1 % 1,4-butanedioldiglycidyl ether

chitosan obtained using these cross-linking agents did not show any significant change under different pH values like the corresponding cross-linking agent alone. Thus, it is confirmed that the different scopes of cross-linking reaction will obviously affect the expansion and biodegradable properties of cross-linked macroporous chitosan membranes discussed in this study.

Enzyme Immobilization

Covalent immobilization of BSA was carried out in this study under various pH values using different initial concentrations of enzymes. The effective immobilization was confirmed and the enzymatic activities of native enzyme and different immobilized macroporous chitosan systems (before and after cross-linking). The properties of immobilized enzyme can be easily understood from experimental results as shown in Table S1 (See Table S1). The relative enzyme activity was determined using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and compared as shown in Table S1 (See Table S1). The percentage of immobilized enzyme (E_{imm}) could be determined (Bautista et al. 1999) by the difference between the relative activity of the native enzyme (r_{nat}) and the relative activity of the filtrate (r_{fil}) in the immobilization process as follows;

$$E_{\text{imm}} = \{(r_{\text{nat}}) - (r_{\text{fil}})/r_{\text{nat}}\} \times 100$$

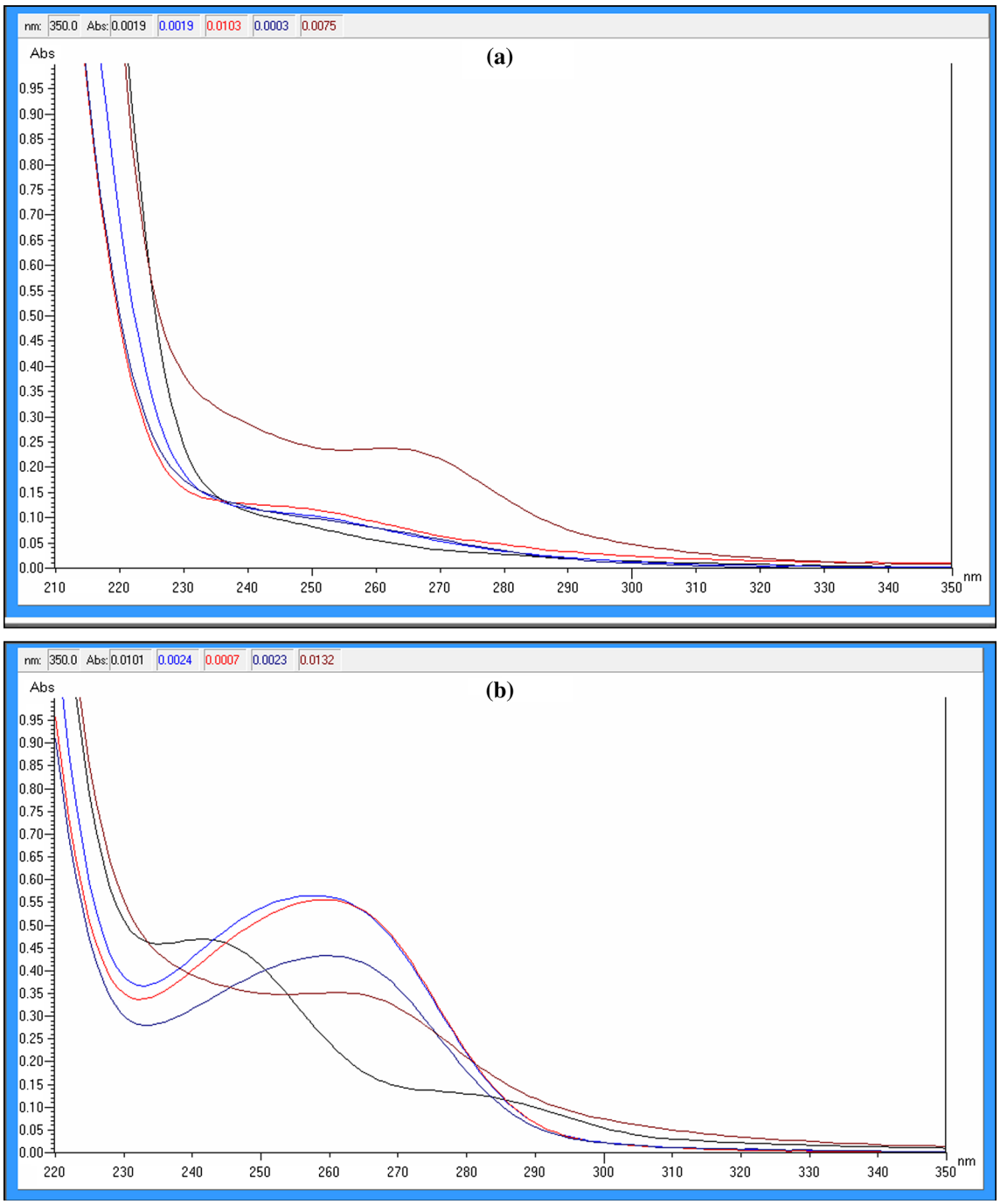

Fig. 4 UV–visible spectra of **a** 1 % 1,4-butanedioldiglycidyl ether; **b** CM15 cross-linked with 1 % 1,4-butanedioldiglycidyl ether

According to the results, the percentage of the immobilized enzymes obtained on cross-linked chitosan membranes is always higher than that of as-synthesized chitosan membranes. From Table S1, it can be also seen that if the initial concentration of enzyme increased, the percentage of immobilization also increased drastically. In the case of 50 ppm BSA, E_{imm} for as-synthesized membrane is 11 %, whereas for cross-linked membrane is 44 %. Similarly in the case of 100 ppm BSA, E_{imm} for as-synthesized membrane is 44 % where as for cross-linked membrane is 56 %. If the initial concentration of BSA was further increased, E_{imm} for as-synthesized membrane is 55 % and for cross-linked membrane is 66 %. Thus, it could be concluded that the cross-linking surface activation of silica-supported chitosan membranes was effective. However, the extent of immobilization is not guarantee enough to obtain

appropriate enzyme activity, because after immobilization, the enzyme practically became inactive also, i.e., it still had some residual activity in filtrate. Thus, it is well known that the active sites of enzyme either involved in its attachment to the support surface or influenced by the steric hindrance of support. Thus, in the case of immobilization process, the covalent immobilization onto membranes is stronger and the actual (total) enzymatic activity was reduced (Bautista et al. 1999). Thus, the enzymatic activity of only immobilized enzyme was considered to compare the results further. The relative activity of native BSA, immobilized onto as-synthesized chitaosan and cross-linked chitosan under various pH ranges for 30 s is shown in Fig. 5. As seen from Fig. 5, both the cross-linked membranes as well as as-synthesized membranes promoted accelerated activation of immobilized enzyme even higher than that of total (actual) activity of native enzyme starting from pH 2 to 8 as observed in this study. But however, the extent of activation of as-synthesized membranes is lower when compared with cross-linked one. But only at pH 2, an interesting observation is connoted that the relative activity of enzyme immobilized onto as-synthesized membrane was found to be higher comparatively than that of both and immobilized onto cross-linked membrane. Among all, enzyme immobilized onto cross-linked membrane displayed lower activity at pH 2. This discrepancy is probably due to steric hindrance of the cross-linked network on the support surface (Bautista et al. 1999) at pH 2. Thus, it is important to understand that for covalent immobilization of enzyme onto the surface of silica support, a treatment of surface activation with some functional organosilane is also necessary (Bautista et al. 1999; Plueddmann 1991) particularly at lower pH. The pH effect as shown in Fig. 5 is a critical

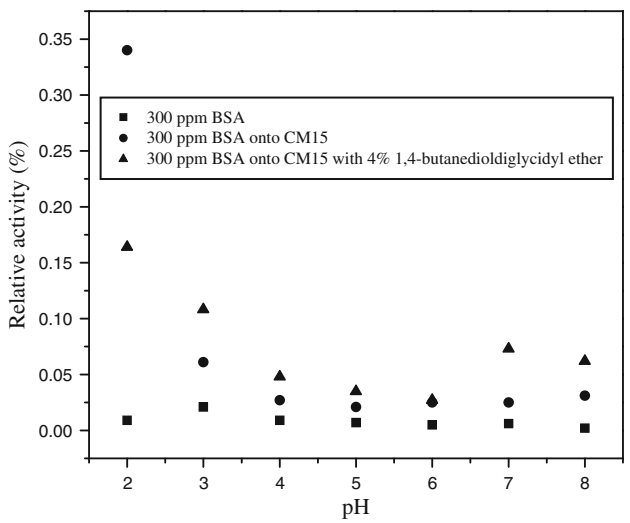

Fig. 5 ABTS relative activity of 300 ppm BSA for 30 s under various pH values before and after immobilization

performance to indicate the stability of immobilized enzyme. As the pH increases, the relative activity decreased and thus the enzymatic activity is stable at low pH only. In the case of native BSA, the relative activity increased from pH 2 to 3, and then gradually decreased on increasing the pH values, whereas in the case of immobilization onto cross-linked membranes, the relative activity gradually decreased from pH 2 to 6 but at neutral pH (7) it suddenly increased and then it decreased at pH 8. But in the case of immobilization onto as-synthesized membrane, the activity gradually kept on decreasing with increasing pH values. Both in the case of native and immobilized onto as-synthesized membrane, the enzymatic activity of BSA sharply decreased from higher value to lower value from pH 2 to 4 and thus the deactivation of enzyme occurred quickly, whereas in the case of cross-linked membrane, the enzymatic activity of BSA gradually decreased, and however, the extent of deactivation is comparatively lower than that of other cases. It is also indicated that the cross-linked membranes are somewhat more stable than that of as-synthesized membranes over a pH range. Thus, it is confirmed that the cross-linking of the membranes definitely increased the enzymatic activity even higher than that of native enzyme upon immobilization. Thus, the pH effect on different systems is quite interesting and confirmed that different mechanisms played an important role in different cases. Perhaps, the most outstanding property of BSA is its ability to bind reversibly with variety of ligands and metal ions. The general mechanism of both cross-linking and immobilization processes can be understood as represented in Fig. 6. Thus, the activation of chitosan membrane was obtained on OH functional group (except glutaraldehyde cross-linking) which is anchored through the cross-linking agent. Covalent immobilization of the enzyme was carried out by the interaction of amino group of BSA enzyme with OH group of the linker formed by cross-linking. However, in the case of glutaraldehyde cross-linked macroporous chitosan membrane, the immobilization may occur on OH group presents in the chitosan unit and not on the cross-linked unit. Thus, it is very clear that the presence of OH group is essential for the immobilization process. However, the presence of silica gel could also enhance such covalent immobilization of enzyme through effective interaction. Thus, in the case of cross-linked membranes, the possible availability of OH functional groups for the coupling of amino group of enzyme definitely increases due to the presence of both silica support and cross-linking agents. Also it is witnessed from the mechanism that the immobilization of enzyme was also obtained on surface of the membrane leaving the pores created by porogen free, and thus the cross-linked membrane provides facility for incorporating some other molecules. Thus, the functional ability of cross-linked membrane is also well established.

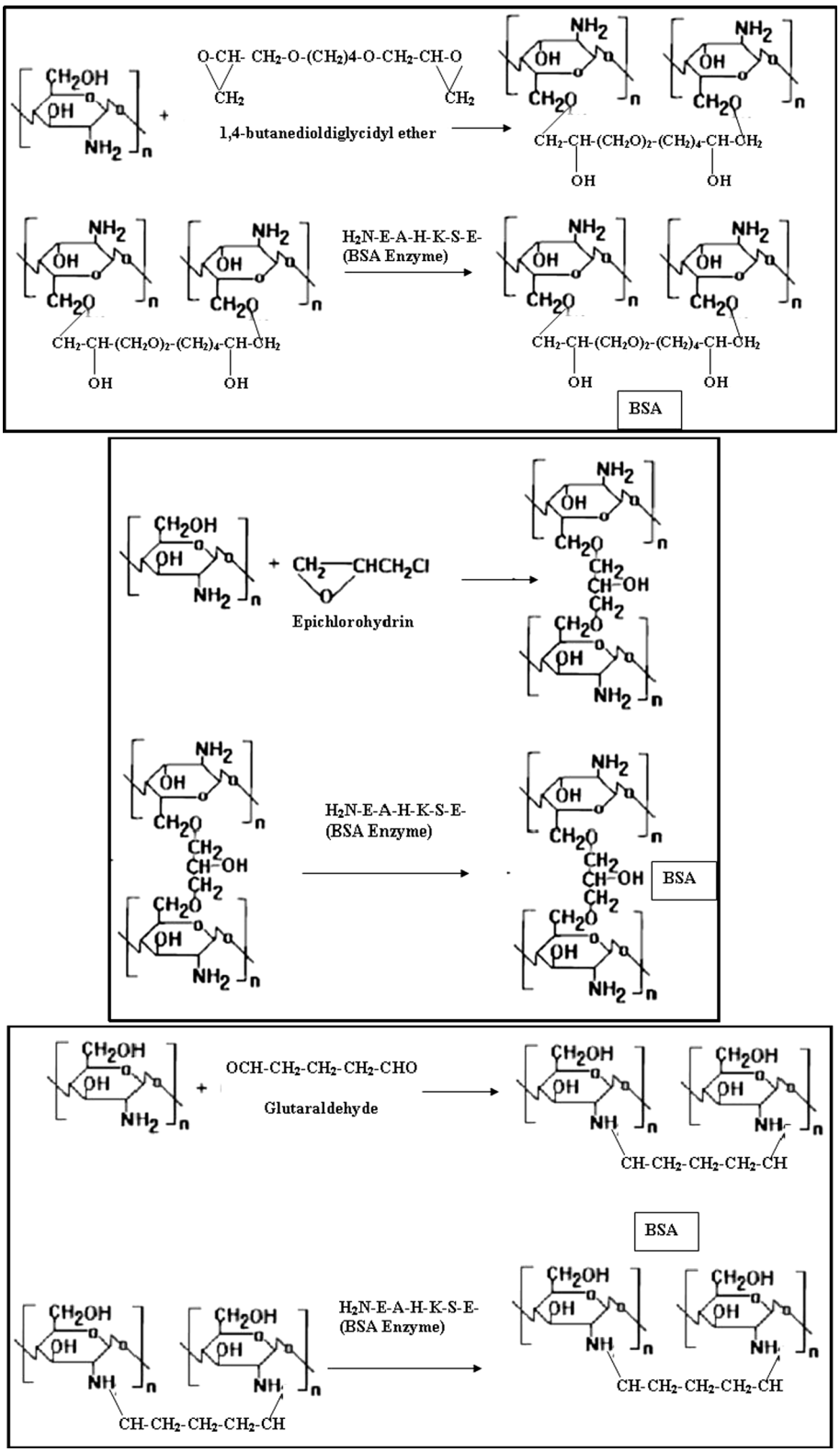

Fig. 6 Schematic representation of cross-linking and enzyme immobilization mechanism

Conclusions

Silica gel-supported macroporous chitosan membranes with good mechanical strength were prepared successfully and reported in this study. The flow rate and porosity of the membranes were affected by both the particle size and weight ratio of the silica porogen. By increasing the weight ratio of silica porogen, the flow rate and porosity also

increased. The activity of the membranes was enhanced by surface modification using various cross-linking agents and effect of various parameters was discussed in detail. The cross-linking of 1,4-butanedioldiglycidyl ether under various pH interestingly provided variation in the observation of results. But other cross-linking agents used in this study did not provide any change upon cross-linking under various pH values. The presence of both NH_2 groups and large particle silica support in cross-linked chitosan membranes could provide large surface for enzyme immobilization. These obtained membranes could serve as an ion exchangers and robust receptors for different guest molecules, and thus provide a wide range of application in various fields. These membranes could also act as both chemically and mechanically stable host molecules for enzyme immobilization and enzyme BSA immobilization was also carried out in this study, and the enzyme immobilized membranes were analyzed in various aspects. Various techniques were employed to demonstrate the formation and activity of macroporous chitosan membranes. The extent of immobilization onto cross-linked membranes is greater than that of as-synthesized membranes and the cross-linked immobilized membranes are more strong and stable than that of immobilized as-synthesized membranes. However, some extent of loss of immobilization was obtained throughout the immobilization process. However, consequently, the immobilization process onto both as-synthesized membrane and cross-linked membrane accelerated the enzymatic activity even to a higher extent than that of total (actual) activity of native enzyme. The relative activity of immobilized enzyme (except pH 2) onto cross-linked membranes is comparatively higher than that of immobilized enzyme onto as-synthesized membranes. Besides to enzyme immobilization, these membranes include synthetic and analytical applications approach for bioremediation of both contaminated soils and water.

We also believe that the increase in weight ratio of silica porogen will definitely alter the pore size also, and thus the number and size of the pores will be easily controlled by varying the amount of silica porogen. So our future study will be further extended to vary the amount of silica gel to obtain different size membranes and to study their activity. Also, the major significance of the membranes obtained using this method is that they can even function as bifunctional material. Thus, we further plan to incorporate heavy metal ions into the various membranes and also study their immobilization activity.

Acknowledgments We thank the National Central University and National Science Council (NSC, (Grant No.: NSC100-2923-E-008-001-MY3), Taiwan, Republic of China (ROC) and Department of Science and Technology (DST, Ref no/S/FST/ESI-101/2010), New Delhi, India, for financial support.

References

Anjali Devi D, Smitha B, Sridhar S, Aminabhavi TM (2005) Pervaporation separation of isopropanol/water mixtures through crosslinked chitosan membranes. J Membr Sci 262:91–99

Asaeda M, Ishida M, Waki T (2005) Pervaporation of aqueous organic acid solutions by porous ceramic membranes. J Chem Eng Jpn 38:336–343

Bautista FM, Bravo MC, Campelo JM, Garcia A, Luna D, Marinas JM, Romero AA (1999) Covalent immobilization of acid phosphatase on amorphous $AlPO^4$ support. J Mol Catal B 6:473–481

Beppu MM, Santana CC (2001) In vitro biomineralization of chitosan. Key Eng Mater 31:192–195

Beppu MM, Arruda EJ, Vieira RS, Santos NN (2004) Adsorption of Cu(II) on porous chitosan membranes functionalized with histidine. J Membr Sci 240:227–235

Beppu MM, Vieira RS, Aimoli CG, Santana CC (2007) Crosslinking of chitosan membranes using glutaraldehyde: effect on ion permeability and water absorption. J Membr Sci 301:126–130

Desai K, Kit K, Li J, Zivanovic S (2008) Morphological and surface properties of electrospun chitosan nanofibers. Biomacromolecules 9:1000–1006

Ge J, Cui Y, Yan Y, Jiang W (2000) The effect of structure of pervaporation of chitosan membrane. J Membr Sci 165:75–81

Hong P-Z, Li S-D, Ou C-Y, Li C-P, Yang L, Zhang C-H (2007) Thermogravimetric analysis of chitosan. J Appl Polym Sci 105:547–551

Iwata H, Saito K, Furusaki S (1991) Adsorption characteristics of an immobilized metal affinity membrane. Biotechnol Prog 7:412–418

Kawai T, Saito K, Lee W (2003) Protein binding to polymer brush, based on ion-exchange, hydrophobic, and affinity interactions. J Chromatogr B 790:131–142

Kuraoka K, Kubo N, Yazawa T (2000) Microporous silica xerogel membrane with high selectivity. J Sol-Gel Sci Technol 19:515–518

Manjubala I, Scheler S, Bössert J, Jandt KD (2006) Mineralisation of chitosan scaffolds with nano-apatite formation by double diffusion technique. Acta Biomater 2:75–84

Nisnevitch M, Firer MA (2001) The solid phase in affinity chromatography: strategies for antibody attachment. J Biochem Biophys Methods 49:467–480

Oh J-T, Kim J-H (2000) Preparation and properties of immobilized amyloglucosidase on nonporous PS/PNaSS microspheres. Enzyme Microb Technol 27:356–361

Okuyama K, Noguchi K, Miyazawa T, Yui T, Ogawa K (1997) Molecular and crystal structure of hydrated chitosan. Macromolecules 30:5849–5855

Plueddmann EP (1991) Silane coupling agents. Plenum, New York

Shentu J, Wu J, Song W, Jia Z (2005) Chitosan microspheres as immobilized dye affinity support for catalase adsorption. Int J Biol Macromol 37:42–46

Singh DK, Ray AR (1998) Characterization of grafted chitosan films. Carbohyd Polym 36:251–255

Suen SY, Lin SY, Chin HC (2000) Effects of spaces arms on Cibacron Blue 3GA immobilization and lysozyme adsorption using regenerated cellulose membrane discs. Ind Eng Chem Res 39:478–487

Uragami T, Matsuda T, Okuno H, Miyata T (1994) Structure of chemically modified chitosan membranes and their characteristics of permeation and separation of aqueous ethanol solutions. J Membr Sci 88:243–251

Wang XP, Shen ZQ, Zhang FY (1998) Pervaporation separation of water alcohol mixtures through hydroxypropylated chitosan membranes. J Appl Polym Sci 69:2035–2041

Wei YC, Hudson SM, Mayer JM, Kaplan DL (1992) The crosslinking of chitosan fibers. J Polym Sci 30:2187–2193

Zakaria Z, Izzah Z, Jawaid M, Hassan A (2012) Effect of degree of deacetylation of chitosan on thermal stability and compatibility of chitosan-polyamide blend. Bioresources 7:5568–5580

Zeng X, Ruckenstein E (1996a) Supported chitosan-dye affinity membranes and their protein adsorption. J Membr Sci 117:271–278

Zeng X, Ruckenstein E (1996b) Control of pore sizes in macroporous chitosan and chitin membranes. Ind Eng Chem Res 35:4169–4175

Zeng X, Ruckenstein E (1998) Cross-linked macroporous chitosan anion-exchange membranes for protein separations. J Membr Sci 148:195–205