Immobilization of Glucose Oxidase in Chitosan Gel Beads

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Received 1 March 2003; accepted 16 June 2003

ABSTRACT: Glucose oxidase was conjugated to the biopolymer chitosan using carbodiimide conjugation. Gel beads were formed from the enzyme–biopolymer complex, and the activity of the immobilized enzyme was compared to the method of encapsulating the enzyme in chitosan. The activity of the immobilized enzyme was found to increase with increasing carbodiimide concentration and time of incubation, to a maximum. Enzyme activity decreased with time as the beads were air-dried. The pH optima of the immobilized enzyme was shifted to a more acidic value compared to that of the free enzyme. The use of carbodiimide chemistry to conjugate glucose oxidase to a chitosan gel was demonstrated. This technique holds promise for the development of immobilized enzymes for applications in medicine, biosensors, and bioprocessing. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 861–866, 2004

Key words: chitosan; gels; enzymes; glucose oxidase; biopolymers

INTRODUCTION

Entrapment of bioactive molecules, such as enzymes, drugs, vitamins, and peptides, in stabilizing matrices has been practiced by the pharmaceutical, food, biomedical, chemical, and waste treatment industries for many years. Although specific requirements are industry and application dependent, general desirable attributes of an entrapment matrix include low cost, ease in handling, abundance, and versatility. Over the past decade, the biopolymer chitosan has attracted interest as a potential matrix for immobilization or controlled release of cells and numerous bioactive compounds.[1,2]

Chitosan is naturally derived (from crustaceans and fungi), biocompatible, well tolerated *in vivo*, and therefore has a great deal of potential for application in the medical and food industries.[3,4] The use of a chitosan gel matrix for the entrapment of proteins and enzymes was investigated by several researchers and found to result in good entrapment efficiencies (in some cases as much as 80%), but also a leakage of protein from the gel bead form.[5–7] Ghanem and Skonberg[7] showed release of the model protein bovine serum albumin (BSA) from chitosan gel beads over several hours. An immobilized enzyme catalyst requires retention of the enzyme in the matrix, so alternative means of immobilizing an enzyme to a chitosan matrix are sought. One potential method is covalent binding of the enzyme to the support matrix using a coupling agent such as gluteraldehyde.[8–10] However, for applications in food and medicine gluteraldehyde is not suitable because it is known to induce cytotoxic reactions attributed to release of byproducts *in vivo*.[11] Carbodiimide has been used as an alternative to gluteraldehyde to link biological molecules to collagen.[12,13] This study describes the use of carbodiimide to couple enzymes to chitosan gel matrices. This method of immobilizing glucose oxidase to chitosan gel matrices has not previously been reported in the literature.

EXPERIMENTAL

Materials

High molecular weight chitosan [MW = 1×10^6, dodecylamine (DDA) 75–85%] was supplied by Aldrich Chemical Co. (Milwaukee, WI). Glucose oxidase, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), tripolyphosphate (TPP), peroxidase, *o*-dianisidine, potassium phosphate, *N*-hydroxysuccinimide (NHS), sodium chloride, sodium phosphate, and D-(+)-glucose were supplied by Sigma Chemical Co. (St. Louis, MO). Glacial acetic acid was supplied by Fisher Scientific Co. (Nepean, Ontario, Canada). All other chemicals were of reagent grade or higher, and were purchased from EM Science (Gibbstown, NJ) or Fisher Scientific (Fair Lawn, NJ).

Ionotropic entrapment of glucose oxidase

Enzyme was entrapped in a 1.5% (w/v) chitosan matrix as follows. Pure glucose oxidase (0.02 g; 157,500 U/g) and 0.30 g of chitosan (high molecular weight)

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Contract grant sponsor: Natural Sciences and Engineering Research Council (NSERC) of Canada.

Journal of Applied Polymer Science, Vol. 91, 861–866 (2004)

were added to 20 mL of 3% (v/v) acetic acid. The mixture was then filtered through two layers of cheesecloth and allowed to sit to degas. The resulting gel was drawn into a 1-mL syringe and extruded through a 21-gauge needle into a 30-mL solution of 15% sodium TPP at a vertical distance of about 10 cm. After extrusion and curing for 10 min, the beads were removed from the solution and homogenized in 2 mL of distilled water. The homogenized beads were subsequently analyzed for glucose oxidase as total protein by the Lowry assay.[14]

EDC conjugation of glucose oxidase to chitosan gel beads

EDC was used to conjugate the carboxyl groups of the enzyme to the amine groups of the chitosan, using NHS as a catalyst, according to methods reported in the literature.[12,15] Briefly, for each trial, 20 mL of a 0.05*M* phosphate-buffered saline solution (pH 5.0) was prepared containing 0.1 mg/mL pure glucose oxidase (157,500 U/g) and varying concentrations of EDC and NHS at a 1 : 0.6 ratio (EDC: 0–300 m*M*, NHS: 0–180 m*M*). Chitosan beads (1 mL, 1.5%, containing no glucose oxidase), prepared by ionotropic gelation, were washed in 20 mL of 50 m*M* PBS (pH = 5.0) for 30 min. The beads were then added to 20 mL of conjugation solution. The chitosan beads were incubated in the glucose oxidase conjugation solution at room temperature for a specified incubation period with occasional gentle swirling. At the end of the incubation period the conjugation solution was removed using a Pasteur pipette. The beads were subsequently washed with 0.1*M* sodium phosphate for 2 h and 2*M* NaCl for 16–18 h. Beads were placed in 2*M* NaCl for overnight storage. Glucose oxidase activity of the beads was measured using the glucose oxidase activity assay.

The effect of air-drying the beads on the activity of immobilized glucose oxidase was also investigated. Beads were made according to the method described above, using a conjugation solution with an EDC concentration of 25 m*M*, NHS concentration of 15 m*M*, and reaction time period of 4 h. The beads were removed from the coupling solution and dried in air for 0–72 h. Glucose oxidase activity of the beads was then measured.

Determination of glucose oxidase activity

The activity of glucose oxidase was measured according to the Worthington method.[16] The reaction velocity was determined from the increase in absorbance at 460 nm resulting from the oxidation of *o*-dianisidine through a peroxidase coupled system. The method is briefly described here. The following reagents were prepared: 0.1*M* potassium phosphate buffer (PPB; pH = 6.0), 1% (w/v) *o*-dianisidine (DNS), peroxidase in water at a concentration of 200 μg/mL, and 18% (w/v) glucose. The assay buffer was prepared by mixing 0.1 mL of 1% DNS into every 12-mL portion of phosphate buffer required and then saturating the solution with oxygen by bubbling with air for 0.5 h, 30 min before use. Test samples were prepared with 2.5 mL of PPB/DNS buffer, 0.1 mL of 200 μg/mL peroxidase, 0.3 mL of 18% glucose, and 0.1 mL of standard solution. The increase in absorbance at 460 nm was recorded over time. Glucose oxidase activity is expressed as the change in absorbance at 460 nm with time. The activity of the glucose oxidase conjugated chitosan beads was tested by placing 1 mL of the conjugated beads in 50 mL of assay buffer gently mixed. Samples of the assay buffer were taken every minute and the absorbance was recorded. All samples were returned to the release medium.

Determination of pH optima

Batch studies were conducted on the glucose oxidase–immobilized chitosan gel beads to determine the effect on the pH optima of the enzyme. The glucose oxidase activity of the free enzyme was determined according to the method described previously, with the pH of the assay buffer adjusted. The pH optima of the glucose oxidase chitosan beads were determined in a similar way, with 1 mL of extruded beads conjugated with glucose oxidase at an EDC/NHS concentration of 50/30 m*M* as described previously. The beads were washed with 0.1*M* sodium phosphate for 2 h and 2*M* NaCl for 16–18 h and stored in NaCl overnight. Each 1-mL aliquot of beads was placed in 50 mL of assay buffer of varying pH and gently mixed. Samples of the assay buffer were taken every minute and the absorbance was recorded. Crude glucose oxidase (6000 U/g) was used for all pH optima experiments done with the free and conjugated enzyme. Experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Ionotropic entrapment of glucose oxidase

Glucose oxidase was entrapped within a chitosan gel matrix by ionotropic gelation of the glucose oxidase–chitosan solution in the counterion solution of sodium TPP at an initial concentration of 157.5 U/mL (or 157.5 U/mg protein) enzyme in chitosan solution (average of three sets of beads). The capture efficiency was calculated according to the following equation:

$$\text{Capture efficiency (\%)} = (AQ/TQ) \times 100 \quad (1)$$

where AQ is the actual quantity of protein in the bead and TQ is the theoretical quantity (i.e., the initial loading concentration).

Good capture efficiencies were obtained using this entrapment method, for which an average value of 64 ± 2.6% was measured. These results compare well with previous work showing 50–60% capture efficiency for BSA and up to 80% in one study.[6,7] The activity of the enzyme-containing beads compared to the free enzyme activity (i.e., the activity of the enzyme added to the solution before bead formation) was also measured. These results indicate that the activity of the beads is about 33% of the free enzyme activity, when expressed as units per mg protein. Thus, although over 60% of the enzyme (by amount of protein) remains in the bead after ionotropic entrapment, the activity is further reduced. This indicates that the enzyme activity may be affected by the immobilization process. Possible explanations for the observed reduction in activity include an internal mass transfer resistance attributed to the chitosan matrix, steric effects, or a shift in optimal conditions required for substrate conversion.

The enzyme activity of ionotropically entrapped glucose oxidase chitosan beads was compared to the EDC-conjugated glucose oxidase chitosan beads. Both were observed to have a lower activity than that of the free enzyme, with the activity of the EDC-conjugated glucose oxidase beads being even lower than that of the ionotropically entrapped enzyme. It was determined that the activity of the coupled enzyme was lower than that of the ionotropically entrapped by about a factor of 10 (activity of ionotropically entrapped enzyme was 0.219 ΔAbsorbance units at 460/min, whereas the activity of the crosslinked enzyme was 0.022 ΔAbsorbance units at 460/min). The basis for comparison was the amount of glucose oxidase added to the conjugation solution, not the amount of glucose oxidase actually attached to the bead, so the low activity is likely a result of limited glucose oxidase actually being conjugated.

Coupling of glucose oxidase to chitosan gel beads

The glucose oxidase enzyme was coupled directly to the chitosan bead matrix by the EDC/NHS reagent. The concentration of the coupling reagent was varied, and the activity of the glucose oxidase containing chitosan beads was measured. Results showing the glucose oxidase activity in the beads as a function of EDC concentration are shown in Figure 1. These beads were made by a process that was different from that of the ionotropically entrapped beads, in that the chitosan gel bead was formed before the addition of the enzyme. The formed beads made from a 1-mL solution of chitosan (50 beads with diameter of 2.9 ± 0.29 mm) were placed in a solution of enzyme and the EDC/NHS conjugation solution. The results of Figure 1 indicate that, as the EDC/NHS solution concentration was increased (in the presence of excess glucose oxidase), the enzyme activity of the resulting beads increased. The relationship is relatively linear, although the enzyme activity appears to increase more rapidly at lower concentrations of EDC (0–50 m*M*) compared to that at the higher concentrations, and a plateau is reached at higher EDC concentrations with a maximum at 200 m*M*. The proposed mechanism of EDC conjugation is the linking of carboxyl groups on the enzyme to free amino groups of the chitosan, a result that supports this concept by showing that at an excess enzyme level, the amount of EDC is limiting. This result can also be used to determine the optimal conditions for glucose oxidase conjugation to ionotropically formed chitosan beads, which is at an EDC concentration of 200 m*M*.

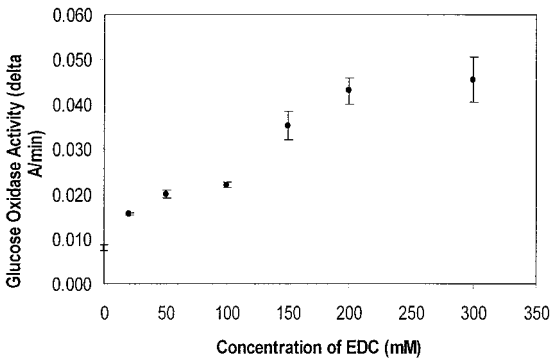

Figure 1 Activity of glucose oxidase immobilized in chitosan gel beads by EDC conjugation, at different EDC concentrations. Each point is the average of three sets of beads. Error bars correspond to SD.

EDC conjugation time

The time of gel bead incubation in the glucose oxidase–EDC/NHS conjugation solution was varied to determine the optimum reaction time period. This experiment was conducted with an EDC concentration of 25 m*M* and NHS of 15 m*M* (the beads formed at the optimal EDC concentration were found to be quite fragile). The conjugation time period was varied from 0.5 to 24 h. Results, shown in Figure 2, indicate that the enzyme activity of the beads increased with increasing conjugation time to a maximum time that was reached at about 12 h. This is somewhat longer than the conjugation time used in most experiments, which is 4–8 h.[15,17] The activity increases much more rapidly with conjugation time initially (0–5 h), with the activity reaching 75% of its maximum value at 5-h incubation. Continuing the reaction past this time period yields only limited gains in activity for increasing reaction time expended.

Effect of drying the glucose oxidase chitosan beads

The final form of the enzyme-containing beads can affect activity, catalyst lifetime, and ease of handling.

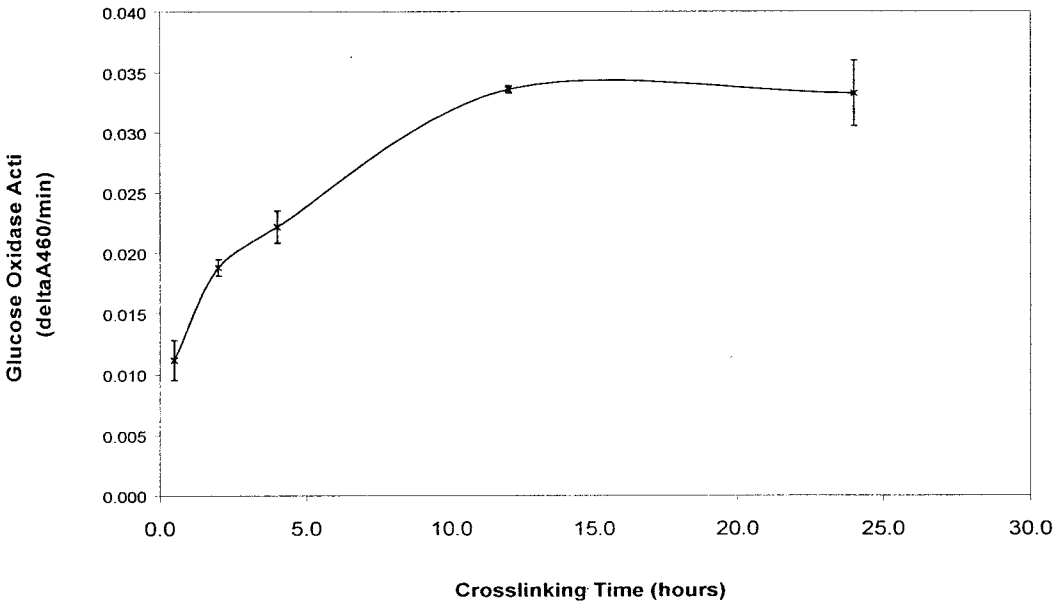

Figure 2 Activity of glucose oxidase immobilized in chitosan gel beads by EDC conjugation, at an EDC concentration of 25 m*M*, conjugated for differing time periods. Results are shown as increase in absorbance with time. Experiment was conducted in triplicate; error bars represent SD.

Gel beads are often more practical to store and handle if preserved in a dry form: drying may also slow down the release of immobilized molecules. The effect of air-drying the beads on the enzyme activity was determined and compared to gel beads. Batches of gel beads were made as indicated previously, and after recovery and rinsing they were then air-dried for differing time intervals. This resulted in considerable shrinkage of the beads from the original size of about 2.9 to 0.5–1 mm. The glucose oxidase activity for each batch of beads was determined as described previously. Results, expressed as enzyme activity, are shown in Figure 3. This figure indicates that the glucose oxidase–containing beads made under identical enzyme loading and conjugation conditions (reaction concentration and time) have varying activities depending on the length of time they were air-dried.

It was observed that the activity of the enzyme decreased with increased drying time and the relationship between drying time and activity was relatively linear. The activity did not appear to reach a minimum level with drying, indicating that there may be a further decrease as drying time is increased beyond 72 h. This decrease in activity may be attributed to the

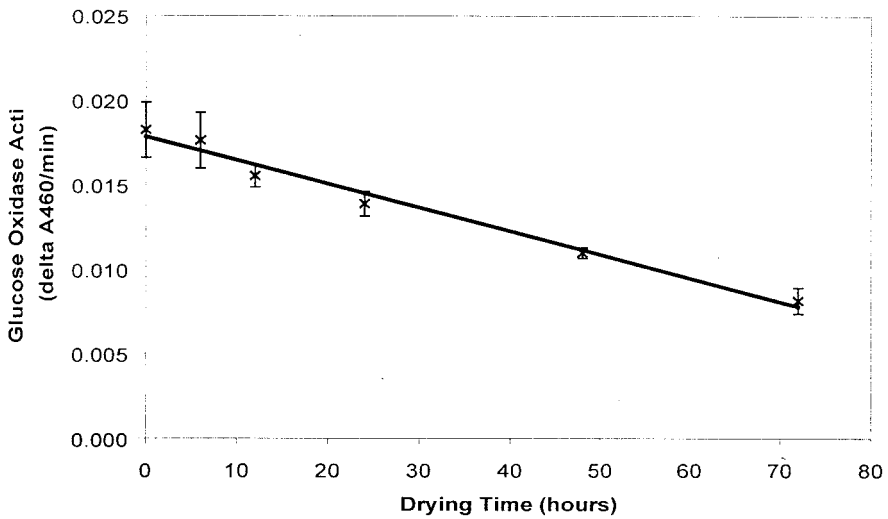

Figure 3 Activity of glucose oxidase immobilized in chitosan gel beads at an EDC concentration of 25 m*M*, incubated for 4 h and air-dried at room temperature for different time periods. Experiment was conducted in triplicate; error bars represent SD.

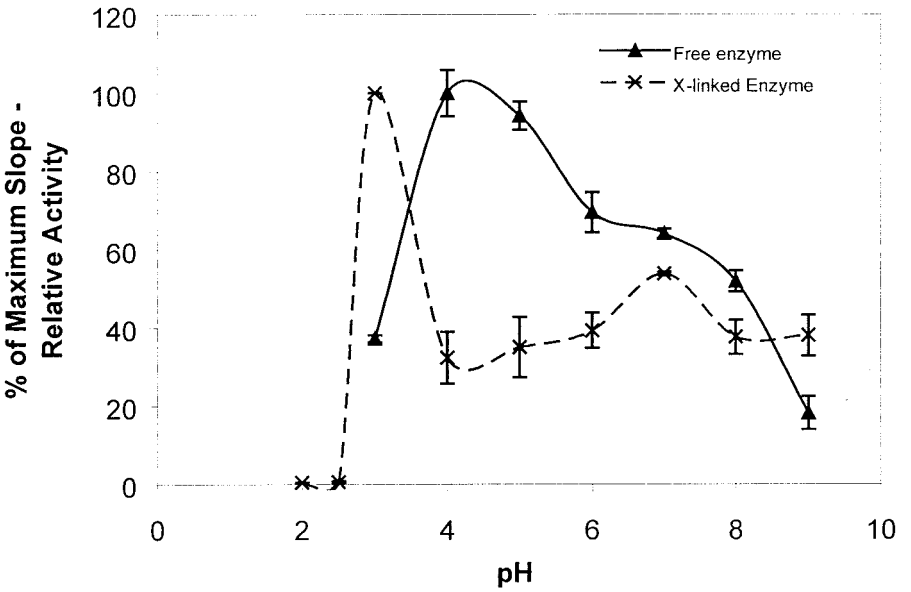

Figure 4 Relative glucose oxidase activity of glucose oxidase immobilized in chitosan gel beads by EDC conjugation (50 m*M* EDC, 30 m*M* NHS, 4 h) at different activity buffer pHs, compared to free enzyme. Each point is the average of four trials; error bars show SD.

water lost from the beads during drying, accompanied by shrinkage and collapsing of the porous gel structure. This hypothesis was tested by determining the time and extent of rehydration of the dried gel beads. Fully dried (dehydrated) beads were rehydrated by placing them in buffer solution and weighing the beads over time, as they absorbed moisture, until a constant weight (rehydrated) was achieved. Results of this test showed that, when dried, the beads were only 24% of their original weight. After rehydration the beads reached a maximum of 34% of their original weight. This rehydration weight was reached 1 h into the rehydration procedure, and remained constant for 50 h. These results indicate that the bead network does not reach its original structure during the glucose oxidase assay and lends evidence to the hypothesis that network collapse and diffusional limitations cause the reduction in enzyme activity that was observed. This result is an important principle when considering the final application of the immobilized enzyme. It is recommended that the beads be used in their hydrated gel form when possible, for maximum enzyme activity.

Determination of pH optima

A shift in optimal operating conditions is often observed for immobilized enzymes and can be an advantage in the final application. Additionally, it has been observed that the pH of surrounding media has an effect on the rate of release of molecules entrapped in a chitosan gel matrix.[7,18] This may be attributable to the swelling characteristics of the chitosan gel, and could be used for pH-triggered drug delivery. A similar concept is extended to an immobilized enzyme system where changes in pH may be used to activate or deactivate the enzyme. With this type of application in mind, the activity of the glucose oxidase–chitosan conjugate beads was measured under different pH conditions and compared to the activity of the free enzyme. The results of this experiment, where enzyme activity as a percentage of the maximum activity is compared to the pH of the assay buffer, are shown in Figure 4. These results were found to be reproducible (results shown are an average of four separate trials). The optimum pH for the immobilized enzyme (pH = 3) was determined to be shifted to the more acidic range compared to the optimum pH for the free enzyme (pH = 4). An enzyme immobilized on a charged support such as chitosan may experience a local pH substantially different from the bulk pH that results from an attraction of oppositely charged ions (in this case, negatively charged particles). A similar trend was observed with the ionotropically entrapped enzyme β-galactosidase. The optimum pH of β-galactosidase was shifted from 7.3 to 6.5 (data not published), indicating this phenomenon is not specific to the EDC-conjugated enzyme. This corresponds to work reported in the literature[9] where immobilization of tannase in chitosan experienced a downward shift in pH optima.

A decrease in the optimum activity range was also observed, given that the peak in activity for the immobilized enzyme was much sharper compared to the broader range for the free enzyme.

CONCLUSIONS

The results obtained in this study indicated that EDC conjugation of the enzyme glucose oxidase to the biopolymer chitosan is a suitable method for formation of immobilized enzyme complexes. Because EDC conjugation does not introduce additional moieties into the enzyme–matrix complex, this method may prove to form more biocompatible immobilized enzymes than gluteraldehyde crosslinked chitosan matrices. EDC-conjugated glucose oxidase chitosan beads may have applications as a biosensor in the determination of glucose and may possibly be used for the controlled release of other medically significant biomolecules, such as insulin for treatment of diabetes mellitus.

Based on the experiments conducted and the results obtained the following conclusions can be made:

- Ionotropic entrapment resulted in a capture efficiency of glucose oxidase consistently over 60%.
- EDC effectively conjugated glucose oxidase to chitosan gel beads. The enyzme activity was found to increase linearly with increased coupling agent (EDC) concentration to a maximum EDC concentration of 200 m*M*.
- The optimum conjugation time for the EDC concentration used (25 m*M*) was found to be 12 h and the activity was found to increase linearly with increased conjugation time until a maximum was reached.
- Gel beads had a higher enzyme activity than that of air-dried beads, and the activity of the enzyme was found to decrease linearly with increasing drying time.
- The optimum pH of the EDC-conjugated enzyme decreased both in value and in range compared to that of the free enzyme activity.

The work shown in this study indicates the very promising application of using EDC to conjugate protein molecules to chitosan, providing a more biocompatible alternative to gluteraldehyde as a coupling agent.

This work was financially supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

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