Preparation and Characterization of α-Amylase Immobilized Inorganic/Organic Hybrid Membrane Using Chitosan as a Dispersant in the Sol-Gel Process

Gyoujin Cho,* Il-Shik Moon, and Jae-Suk Lee†
Department of Chemical Engineering, Suncheon National University, Suncheon, Chunnam, 540-742 Korea
†*Department of Material Science and Engineering, Kwangju Institute of Science and Technology (K-JIST), Kwangju, 506-303 Korea*

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A new method for immobilizing α-amylase in a hybrid inorganic/organic membrane has been developed for the purpose of potential applications as a membrane bioreactor in aqueous phase. It has been found that chitosan can act as an enzyme-stabilizing host and a dispersant which can homogeneously mix an inorganic (SiO_2) phase and an organic (polymer) phase. The optimum conditions in preparing the membrane were determined. The resulting microscopically homogeneous membranes were characterized by FT-IR, TGA, and water stability. It was found that the enzyme-incorporated membrane showed a very stable activity for the period of our experimental set-up (30 days).

In recent years, many studies have been reported on the preparation of immobilized enzymes.[1,2] There are three common methods for immobilizing an enzyme on insoluble matrices: 1) covalent binding to the matrices, 2) physical and ionic adsorptions to the matrices, 3) entraping in the matrices. Among the matrices for immobilization, inorganic glasses composed of oxides of boron, aluminum, silicon, titanium, zirconium, vanadium, etc. can offer rigid, amorphous, three-dimensional structures which often have excellent optical clarity.[3] Therefore, they have been considered as host materials for the incorporation of various organic, and inorganic materials. However, the processing temperatures of most commonly used inorganic glasses often exceed the decomposition temperatures of common organic materials and the denaturation temperatures of living organisms, severely limiting their application.

As an attractive alternative, inorganic and modified inorganic/organic hybrid glasses can often be prepared *in situ* by low temperature polymerization of appropriate monomers in a sol-gel process.[4] The sol-gel method opened a new way in preparing host matrices to encapsulate unique guest molecules. Recently, we have reported the use of the sol-gel method to offer thermal stability to nonlinear optical organic chromophores in silica matrices.[5] As an obvious extension of the sol-gel method, it is of interest to investigate whether enzymes can be incorporated in the glass matrices without losing any viability and activity. The enzyme incorporated glass membrane may have potential to be used in the field of membrane bioreactors.[6] However, the brittleness of the glasses is a major obstacle in their adoption as membranes. Fortunately, in an adaptation of the method of interpenetrating polymer network (IPN), both the flexible and microscopically homogeneous morphology of organic/inorganic hybrid membranes can be prepared.[7] In this study, an organic/inorganic hybrid membrane is prepared from both organic polymer and inorganic SiO_2 phases. The organic polymer phase is obtained from *in situ* copolymerization of both methylmethacrylate (MMA) and 3-(trimethoxysilyl) propylmethacrylate (TSM). The inorganic SiO_2 phase is obtained from a sol-gel hydrolysis of both tetraethyl orthosilicate (TEOS) and TSM. Chitosan is used as a dispersant for the membrane matrix. Since it is well known that chitosan can act as a biocompatible host to provide cell viability and activity,[8] it may act not only to disperse the organic phase in the matrice but also provide a friendly environment to the guest enzyme (α-amylase).

In this paper, we wish to report the optimum condition in the construction and characterization of the microscopically homogeneous hybrid membrane in which α-amylase has been immobilized using FT-IR and canning electron microscopy (SEM). Both water stability and enzyme activity of the resulting membrane have been determined as well.

The immobilization of enzyme was carried out using a modified sol-gel procedure[9] in preparing the membrane except that α-amylase (Termamyl® from NOVO Co.) was added into the chitosan solution. For our experiments, the optimum weight ratios between the reagents in constructing the membrane were TEOS (3): MMA (4.2): TSM (1): chitosan (1) while the mole ratios between TEOS, water, acetic acid, and ethanol were kept to 1 : 314 : 9.5 : 128. The optimum loading condition of the enzyme was obtained when the enzyme was loaded to 0.1 mL in 20 mL of the prepared optimum sol-gel solution with the composition mentioned above. In this final sol-gel solution, the *in situ* copolymerization of TSM and MMA occurs under acidic condition at 50°C. Once polymerization had occured, the resulting gel was allowed to cure at 50°C for another 24 h. During this time, no macro- or microphase separation was observed, and there were no shrinkage and separation from the walls of the teflon dish which contained the reaction mixture. After curing, the clear and flexible membrane can be easily pulled away from the dish for further characterization. However, when the sol-gel processing started at 50°C without initial standing at room temperature or the use of chitosan, phase separation was did observed. Based on those results, we speculate that when the free radical copolymerization of TSM and MMA is fast with respect to the sol-gel reaction rate, phase separation would occure during the polymerization. Furthermore, the ratios of TEOS, MMA, TSM, and chitosan are a key factor to tailor the morphology and obtain the microscopically homogeneous membrane.

The FT-IR spectrum of the membrane is charactrized by three intense bands, two in the carbonyl stretching regions at 1717 cm^{-1} and 1690 cm^{-1} for copolyMMA-TSM and chitosan respectively, and one in the Si-O-Si stretching region at 980~1100 cm^{-1}. The carbonyl stretching of copolyMMA-TSM was absent from simple chitosan/silica membrane. *In situ* copolymerization of TSM and MMA at 50°C in the gel can be followed by inspecting the

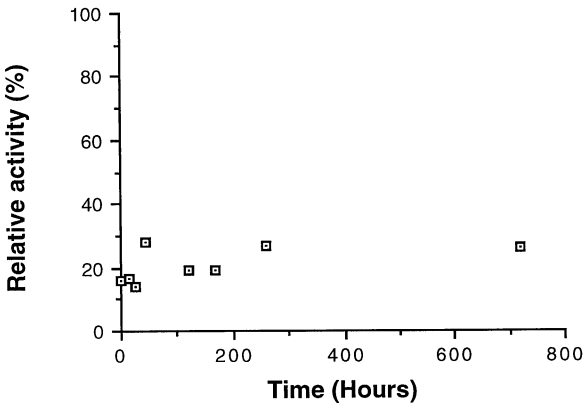

Figure 1. Storage stability of immobilized enzyme at 25 °C.

carbonly stretching regions in the IR spectrum: with heating at 50 °C, the carbonyl peak appeared at the higher energy region (1717 cm^{-1}) but the carbonyl peak of the same membrane prepared without heating showed at 1690 cm^{-1}, coinciding with the peak due to chitosan. This observation indicates that at 50°C the copolymerization of TSM and MMA can be initiated in the gel without phase separation.

Since our interest is in developing a membrane bioreactor in aqueous phase, we tested the stability of the resulting membrane in aqueous solution. The hybrid organic/inorganic membrane was slightly swollen in water, but the weight loss determined by re-drying the swollen membrane was less than 5% for all samples tested. On the other hand, the membrane prepared from only chitosan/silica was completely destroyed in the aqueous phase. This excellent water stability of the TSM/MMA/chitosan/SiO_2 membrane may be due to the hydrophobicity of the copolymer in the membrane.

The enzyme activity was estimated by measuring the amount of maltose produced enzymatically, using starch as a substrate with the DNS method.[10] For the native enzyme, the amount used was calculated based on the same amount of immobilized enzyme in the prepared membrane. The reaction of both native enzyme and immobilized enzyme were carried out in the mixed solution of 1 mL of starch solution for 3 minute at 25 °C. When the immobilized enzyme was used, a piece of the membrane (size = 1 cm^2) was immersed in the reaction solution. All the reactions were carried out without agitation. The relative activity for the immobilized enzyme was calculated based on by: Relative activity (%) = (100 x A) / B where A is the amount of maltose produced by the immobilized enzyme, and B is the amount of the maltose produced by native enzyme.

The water storage stability of the immobilized enzyme as a function of time is plotted in Figure 1. The results show that the activity of immobilized α-amylase is very stable for the storage period of our experimental set-up (30 days). This excellent stable activity may be due to the enhancement of enzyme entrapment by the crosslinking of siloxane, and to the presence of the bio-compatible component chitosan. Although the membrane morphology, as shown in Figures 2 and 3 respectively for the surface and cross-section of the membrane, is revealed by SEM to be a well dispersed and typical symmetric membrane, the morphology of the enzyme-immobilized membrane was strongly affected by the amount of enzyme loading.

In summary, we have prepared water stable α-amylase immobilized organic/inorganic hybrid membranes with

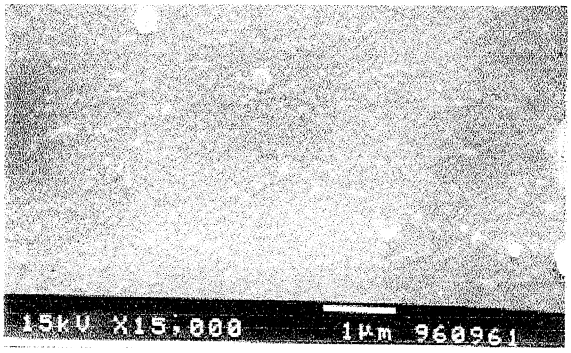

Figure 2. Scaning electron microcopy of surface morphology of enzyme immobilized membrane.

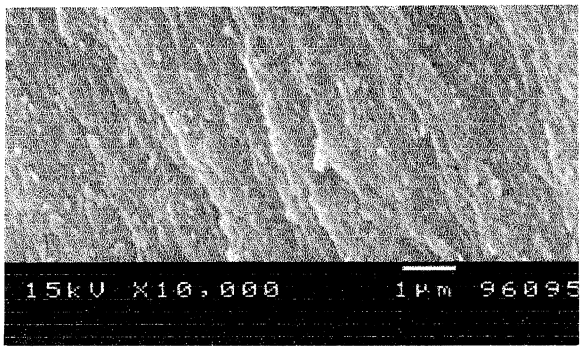

Figure 3. Scaning electron microscopy of cross sectional morphology of enzyme immobilized membrane.

incorporated chitosan by using the *in situ* copolymerization of TSM and MMA in the gel phase. In this process, chitosan acts as a dispersant to homogeneously disperse TSM and MMA in the sol-gel solution. Without chitosan, the microscopically homogeneous water stable membrane could not be prepared. In addition, we have shown that α-amylase in the membrane remained viable. The membrane showed stable activity for 30 days in water storage at 25 °C. The morphology of the membrane showed a microscopically homogeneous and typical symmetry from the SEM investigations.

References and Notes

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9 A series of chitosan entrapped hybrid membranes were prepared by adding an ethanol/tetraethoxyl orthosilicate (TEOS) / 3-(trimethoxysilyl)propyl methacrylate (TSM)/MMA mixture to a water/acetic acid (10 vol%) / chitosan solution with stirring. The free radical initiator for the vinyl copolymerization of TSM and MMA was AIBN, added at 0.01 mol ratio with respect to the loaded TSM. The chitosan acts as a dispersant forming homogeneous solutions upon mixing. The samples were purged with nitrogen and stood at room temperature until the gelation. After the gelation, the sample was placed in a 50 °C dry bath to complete the polymerization and SiO_2 hydrolysis.
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