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Fabrication of amphiphilic copolymeric gels with enhanced activity of immobilized enzymes in organic media

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ABSTRACT: Novel amphiphilic copolymeric gels were developed to immobilize lipase. NIPA-*co*-PEGMEA gels were prepared by copolymerizing *N*-isopropylacrylamide (NIPA) as a thermosensitive and amphiphilic component and poly(ethylene glycol) methyl ether acrylate (PEGMEA) as a hydrophilic component in aqueous media. The gels can absorb organic solvents at temperatures higher than the lower critical solution temperature owing to the thermosensitive and amphiphilic properties of poly(NIPA). The lipase immobilized within the NIPA-*co*-PEGMEA gel, which had a NIPA : PEGMEA composition of 950 : 50 mol/m^3, successfully catalyzed the esterification of oleic acid and ethanol without loss of activity during repeated use within 20–40°C. The activity of the immobilized lipase was considerably higher than that of free lipase. The NIPA-*co*-PEGMEA gels provide a structure that allows the immobilized lipase to work actively in an aqueous environment and with the dispersed state of the lipase in the gels. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41905.

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INTRODUCTION

Enzymes catalyze specific reactions under ambient temperature and atmospheric pressure and are widely applied for organic synthesis (e.g., polyhydroxybutyrate,[1] *N*-substituted acrylamide,[2] lubricants,[3,4] biodiesel,[5,6] and so on), environmental detoxification,[7,8] medical care,[9,10] and so on. In aqueous media, enzymes generally dissolve and exhibit good catalytic activity, whereas in organic media, they exhibit poor catalytic activity, because in organic media enzymes tend to aggregate and most of their active sites are confined inward.[11] The presence of a small optimal amount of water in organic media can increase the enzymatic activity.[12–14]

Enzymes have been successfully immobilized on various water-insoluble supports, such as inorganic materials, synthetic polymeric gels, and natural macromolecules, through physical adsorption, covalent bonding, microencapsulation, and matrix entrapment.[15,16] Immobilized enzymes can be used repeatedly or continuously in a variety of reactors. A suitable support can improve the activity and stability of immobilized enzymes in organic media owing to the dispersion of enzymes[11] and a suitable amount of water[17] in the support.

Synthetic polymeric gels are used as immobilization carriers and they allow substrate molecules and reaction products to easily diffuse in and out. To improve the activity of enzymes and the apparent reaction rate, functional polymeric gels have been applied for the immobilization of enzymes as follows. Thermosensitive *N*-isopropylacrylamide (NIPA) gels switch the activity of immobilized enzymes on and off in response to temperature.[18,19] The NIPA gel is a representative of thermosensitive gels and possesses a lower critical solution temperature (LCST) of around 33°C in water;[20] it exhibits hydrophilicity and hydrophobicity at temperatures lower and higher than the LCST, respectively. Thermosensitive and pH-sensitive gels comprising NIPA and itaconic acid improved the pH stability of immobilized enzymes.[21] A nanophase-separated amphiphilic network comprising hydrophilic 2-hydroxyethyl acrylate and hydrophobic dimethylsiloxane yielded greater activity of immobilized enzymes than that of free enzymes.[22] Thus far, there are few reports of functional polymeric gels applied for immobilization of enzymes.

This study aimed to develop novel functional polymeric gels for the immobilization of enzymes to achieve improved activity of immobilized enzymes for reactions in organic media. We

propose NIPA gels copolymerized with hydrophilic poly(ethylene glycol) methyl ether acrylate (PEGMEA), that is, NIPA-*co*-PEGMEA gels. The amphiphilic property of the NIPA hydrogel/organogel that absorbs water/organic solvents at temperatures below/above the LCST, respectively, provides the following features. During the gel preparation, the use of aqueous media achieves the immobilization of enzymes dispersed within the resultant NIPA hydrogel. When subjected to a reaction in organic media, the NIPA organogel allows the hydrophobic substrate molecules and reaction products to diffuse in and out. The copolymerized PEGMEA provides an aqueous environment and/or a hydrophilic channel locally in the gels, which can improve the activity and stability of the immobilized enzymes and easily expel water (a product yielded by esterification) out of the gels.[22,23] The studied model enzyme reaction was the esterification of oleic acid with ethanol into ethyl oleate catalyzed by lipase. Lipases are part of the hydrolase family that act on carboxylic ester bonds and can catalyze versatile reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis.[24] NIPA-*co*-PEGMEA gels that entrapped lipase with various monomer concentrations were synthesized by free radical polymerization in aqueous media. The swelling and reaction properties of gels with immobilized lipase were investigated.

EXPERIMENTAL

Reagents

NIPA (primary monomer), PEGMEA (secondary monomer), *N*,*N*′-methylenebisacrylamide (MBAA; cross-linker), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; accelerator), and ammonium peroxodisulfate (APS; initiator) were used to form gels. The NIPA was kindly supplied by KJ Chemicals Co. Lipase PS Amano SD (Amano Enzyme) was used as the enzyme and comprised approximately 10 wt % lipase from *Burkholderia cepacia* and 90 wt % dextrin. Oleic acid and ethanol were used as the substrates. All chemicals were used without further purification.

Preparation of Gels

The NIPA-*co*-PEGMEA gels were synthesized by free radical copolymerization. A monomer aqueous solution containing NIPA, PEGMEA, MBAA, TEMED, and lipase and an initiator aqueous solution containing APS were prepared. Each solution was maintained at 10°C and purged with nitrogen gas for 1 h. Next, the initiator aqueous solution was added to the monomer aqueous solution, and polymerization was conducted in an acrylic well plate with an inner diameter of 5 mm and a depth of 2 mm at 10°C for 24 h under a nitrogen atmosphere. The final concentrations of NIPA, PEGMEA, MBAA, TEMED, and APS were 950, 50, 200, 40, and 4 mol/m^3 in the pregel solution, respectively, and Lipase PS Amano SD was 64 kg/m^3. The gels were prepared under various monomer concentrations of NIPA : PEGMEA = 1000 : 0, 990 : 10, 950 : 50, 900 : 100, and 800 : 200 mol/m^3. The resultant gels were washed with water; the lipase (protein) in water after the washing was not detected by a Lowry method. Then, the gels were dried at room temperature.

Measurements of Swelling Diameter

Disk-shaped gel samples in the dry state were initially immersed in water at 20°C. The swelling diameters of the gels were measured with a digital camera. The temperature was then increased in increments from 20 to 60°C, and the diameter, at equilibrium, was measured at each temperature.

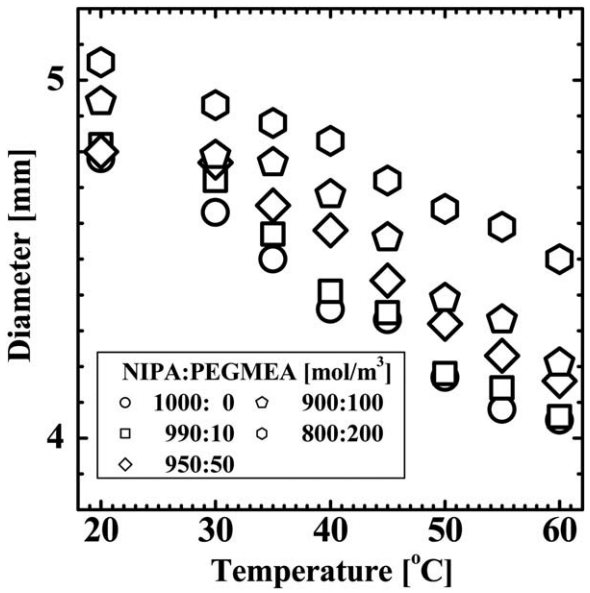

Figure 1. Swelling diameters of disk-shaped NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA in water as a function of temperature.

Disk-shaped gel samples in the dry state were immersed in an enzymatic reaction solution comprising 10 g oleic acid and 1 g ethanol at 40°C, and the diameters of the samples were measured over time.

Lipase-Catalyzed Reactions

The activity levels of the lipase immobilized within the gels were determined by a typical batch method. A portion of dry gels (20 pieces) and 10 g oleic acid (35.4 mmol) were mixed in

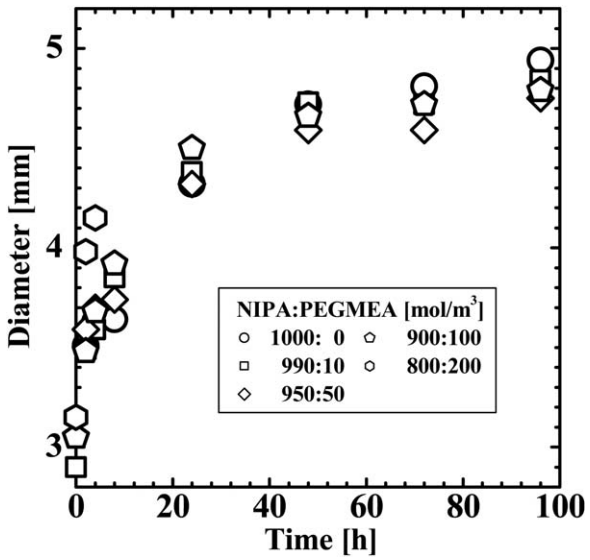

Figure 2. Time courses of swelling diameters of disk-shaped NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA when subjected to immersion in an enzymatic reaction solution (10 g oleic acid and 1 g ethanol) at 40°C.

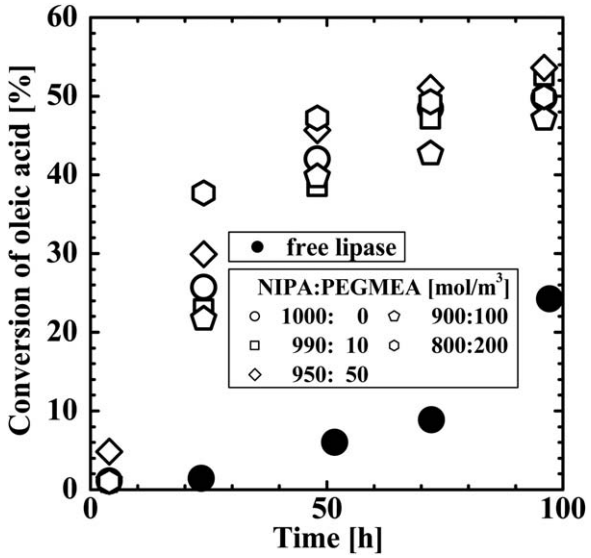

Figure 3. Conversion of oleic acid into ethyl oleate as catalyzed by free lipase and immobilized lipase within NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA at 40°C over time.

a vial. The vial was then left to stand for a day at 40°C to swell the gels. Next, 1 g ethanol (21.7 mmol) was added to the vial, and the vial was then placed in a water bath at a given temperature (20, 30, 40, or 50°C) and was shaken throughout the experiments. An aliquot (0.04 g) of the solution was taken at a specified time and diluted with methanol (5 g). The concentrations of oleic acid and ethyl oleate in the sample solution were measured by gas chromatography, and the conversion of oleic acid into ethyl oleate was calculated. The gels were immersed in a new reaction solution after the experiment, and the reaction was then repeated.

Reactions using free lipase (50 mg) were also carried out, wherein each experimental system contained equal total dosages of lipase.

RESULTS AND DISCUSSION

Swelling Properties

Figure 1 shows the temperature dependence of the swelling diameter of the disk-shaped NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA in water. The diameter of the NIPA gel decreased with increases in temperature owing to the hydrophilic/hydrophobic transition of poly(NIPA). The diameter and the LCST of the NIPA-*co*-PEGMEA gels increased with an increase in the concentration of PEGMEA (hydrophilic comonomer). The swelling patterns of the NIPA-*co*-PEGMEA gels were similar to the results of previously conducted studies on NIPA gels copolymerized with hydrophilic components.[25–27]

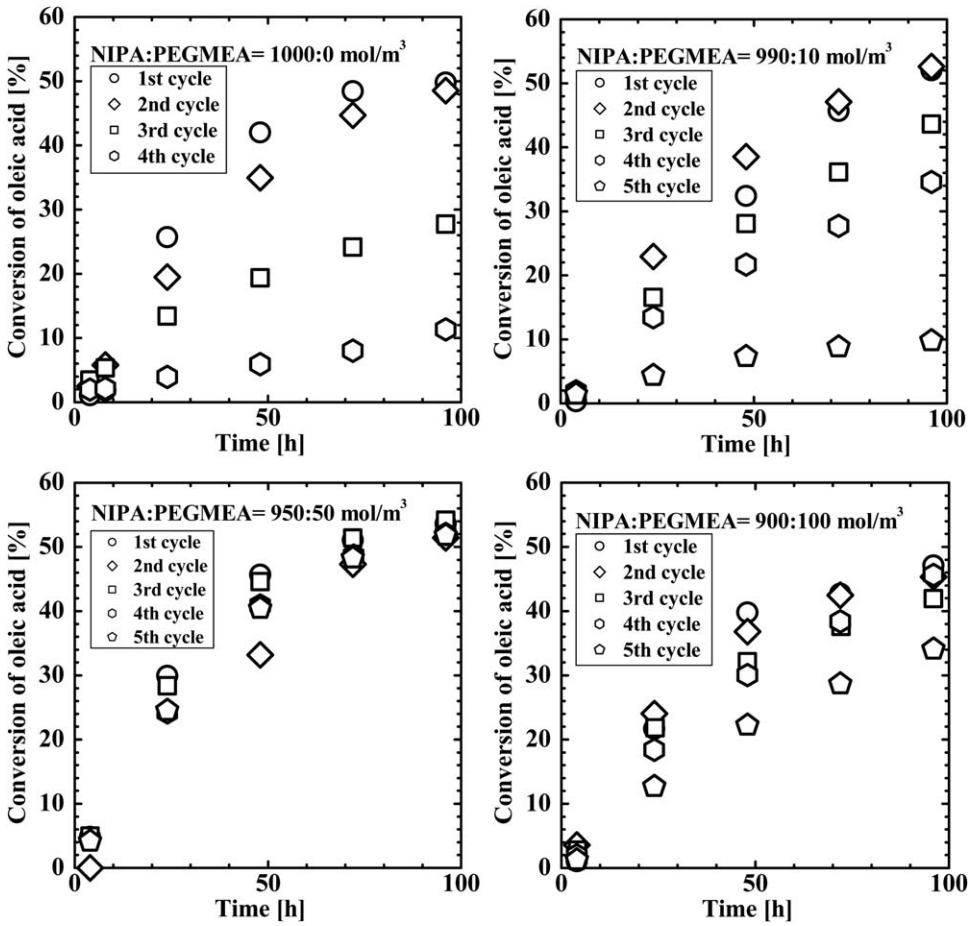

Figure 4. Conversion of oleic acid into ethyl oleate with repeated reaction cycles as catalyzed by the immobilized lipase within NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA at 40°C over time.

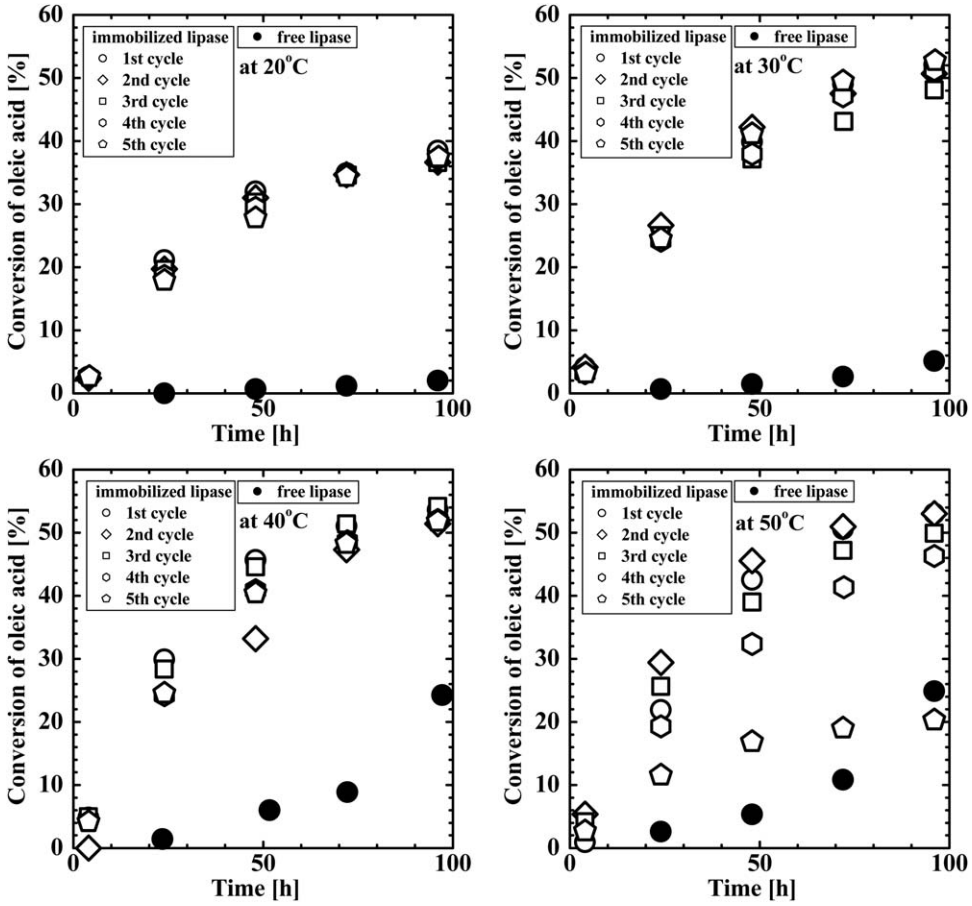

Figure 5. Conversion of oleic acid into ethyl oleate with repeated reaction cycles as catalyzed by free lipase (just one cycle) and immobilized lipase (five cycles) within a NIPA-*co*-PEGMEA gel (NIPA : PEGMEA = 950 : 50 mol/m^3) at given temperature over time.

The NIPA-*co*-PEGMEA gels swell and shrink reversibly when the temperature is alternatively decreased and increased.

Figure 2 shows the time course of the swelling diameters of the disk-shaped NIPA-*co*-PEGMEA gels after the gels in the dry state were immersed in the enzymatic reaction solution (10 g oleic acid and 1 g ethanol) at 40°C. The diameter of the NIPA gel increased with time, and swelling equilibrium was attained in approximately 40 h. The NIPA gel can absorb an organic solvent (e.g., oleyl alcohol[28]) at temperatures higher than the LCST. The swelling patterns of the NIPA-*co*-PEGMEA gels were similar to that of the NIPA gel. The NIPA : PEGMEA = 800 : 200 mol/m^3 gel had the fastest rate of swelling among the gels tested, and spontaneously broke into pieces at 8 h. The destruction of the gel was attributed to the increase in the internal pressure of the gel that absorbed and retained water produced by the simultaneous enzymatic reaction during the swelling process.

Enzymatic Reaction Properties

Figure 3 shows the percent conversion of oleic acid into ethyl oleate at 40°C over time, as catalyzed by free lipase and lipase immobilized within NIPA-*co*-PEGMEA gels with various concentrations of NIPA and PEGMEA. The conversion for the free lipase increased substantially at 72 h and stabilized at 52% for a long duration (200 h); the large increase was because the water produced by the esterification enhanced the lipase activity. The conversion of the lipase immobilized within the NIPA-*co*-PEGMEA gels became approximately 50% at 96 h. These results suggest that the NIPA-*co*-PEGMEA gels provide a structure that allows immobilized lipase to work actively within an aqueous environment and the dispersed state of the lipase in the gels. The NIPA : PEGMEA = 800 : 200 mol/m^3 gel had the fastest rate of enzymatic reaction because of the fast rate of swelling (absorbing substrates); however, the gel broke apart during the enzymatic reaction, as mentioned earlier. The NIPA-*co*-PEGMEA gels containing less than 100 mol/m^3 PEGMEA are promising.

The recyclability of the immobilized lipase within the NIPA-*co*-PEGMEA gels was examined, and the results are shown in Figure 4 (fifth cycles were confirmed). The activity of lipase immobilized within the NIPA gel and the NIPA : PEGMEA = 990 : 10 mol/m^3 gel decreased significantly with the repetition of reaction cycles, while the lipase immobilized within the NIPA : PEGMEA = 950 : 50 mol/m^3 gel was found to catalyze with a certain level of activity through repeated use. As a result, the hydrophobic environment within the gel generated by poly(NIPA) could make lipase denature and/or deactivate, and the PEGMEA produces an aqueous environment locally in the gels, which improves the stability of the lipase. The performance (the conversion and recyclability) of the NIPA : PEGMEA = 950 : 50 mol/m^3 gel was better than that of the NIPA : PEGMEA = 900 : 100 mol/m^3 gel.

Affleck *et al.*[13] reported that the presence of a small optimal amount of water in organic media yielded greater structural flexibility of an enzyme's active sites that increased activity; however, an excessive amount of water produced a minor conformational change that decreased the activity. Murakata *et al.*[18] reported that the immobilized enzyme within the NIPA gel was most active for esterification in organic medium when the amount of water in and around the gel was suitable. Therefore, the NIPA : PEGMEA = 950 : 50 mol/m^3 gel must provide an optimal aqueous environment for immobilized lipase, although the amount of water in the gel was not measured.

Figure 5 shows the effect of temperature on the performance of the free lipase and the immobilized lipase within the NIPA : PEGMEA = 950 : 50 mol/m^3 gel. The activity of the immobilized lipase in the first cycle was relatively high at 30–50°C and low at 20°C. The immobilized lipase exhibited good recyclability without activity loss at 20–40°C, while its activity became significantly poorer in the fifth cycle at 50°C. This behavior arises because of the nature of lipase. As shown in Figure 5, the activity of the free lipase was relatively high at 40 and 50°C and low at 20 and 30°C. The conversion for the free lipase at 20, 30, 40, and 50°C was 24, 45, 52, and 38% for a long duration (200 h), respectively. Our results were supported by the supplier's catalog; from the catalog, it was found that Lipase PS Amano SD is more active at higher temperatures in the range 35–70°C and is fully stable at temperatures less than 40°C.

CONCLUSIONS

NIPA-*co*-PEGMEA gels were developed by free radical copolymerization in aqueous media for the immobilization of enzymes. These gels can absorb organic solvents at temperatures higher than the LCST owing to the thermosensitive and amphiphilic properties of poly(NIPA). The lipase immobilized within the NIPA : PEGMEA = 950 : 50 mol/m^3 gel successfully catalyzed the esterification of oleic acid and ethanol without loss of activity during repeated use within 20–40°C. The activity of the immobilized lipase was considerably higher than that of free lipase. The NIPA-*co*-PEGMEA gels provide a structure that allows immobilized lipase to work actively in an aqueous environment and with the dispersed state of the lipase in the gels.

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