Poly(glycidyl methacrylate)-Polystyrene Diblocks Copolymer Grafted Nanocomposite Microspheres from Surface-Initiated Atom Transfer Radical Polymerization for Lipase Immobilization: Application in Flavor Ester Synthesis

Bunyamin Karagoz,[†] Gulay Bayramoglu,*[,‡] Begum Altintas,[‡] Niyazi Bicak,[†] and M. Yakup Arica[‡]

Department of Chemistry, Istanbul Technical University, Maslak 34469, Istanbul/Turkey, and Biochemical Processing and Biomaterial Research Laboratory, Faculty of Arts and Sciences, Gazi University, Teknik Okullar 06500, Ankara/Turkey

Functional hairy poly(styrene-*b*-glycidylmethacrylate) (P(S-GMA)) brushes were generated by grafting from bromoacetylated poly(styrene-divinylbenzene) (P(S-DVB)) microspheres via surface-initiated atom transfer radical polymerization (SI-ATRP). Two different approaches for the covalent immobilization of lipase onto microspheres were studied for the first time: (1) direct immobilization of lipase to the polymer brushes via their epoxy groups, and (2) immobilization of lipase via glutaraldehyde coupling after attachment of a spacer arm (hexamethylendiamine (HMDA)) to the polymer brushes. The covalent immobilization of the lipase on microspheres after spacer-arm attachment and glutaraldeyhde coupling was found to be the more effective than the direct binding method. In this case, a maximum value of the immobilized enzyme activity 498.5 U g^{-1} was found with an enzyme loading of 27.6 mg per gram of support. Thermal and storage stabilities increase upon immobilization on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres. Finally, esterification reactions have been performed to produce ethyl acetate and isoamyl acetate in a solvent-free system and in n-hexane using lipase-immobilized P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres. The immobilized lipase was effectively reused in successive batch runs in a solvent-free system for isoamyl acetate synthesis, and only 21% activity was lost after 10 cycles.

Introduction

Decoration of solid supports with functional hairy grafts is a new avenue leading to more efficient functional materials.[1−3] The use of the hairy grafts as carriers for biocatalysts provides faster reactions due to partial mobility of the graft chains.[4] Compared with the common beaded polymers possessing directly attached functional groups to the surfaces, the hairy structures ensure quasi homogeneous reaction conditions.[5−7] Considering insolubility of the microsphere core and miscibility of the graft chains in the solvents, the hairy graft structures are easy to isolate and possess fast reaction abilities. Various techniques, such as grafting-onto, grafting-through, and grafting-from approaches, have been studied to produce such structures.[4] The latter technique is mostly preferred, because it provides better control of the chain growth and avoids homopolymer formation. This can be achieved successfully by using controlled/living radical polymerization techniques. Among controlled living polymerization techniques, surface initiated-atom transfer radical polymerization (SI-ATRP) is an especially fruitful process which is applicable in grafting even from solid particle surfaces.[1,8−10] Polymer brushes have been used to immobilize proteins with a wide range of molecular weights, overall charges, and functions. For example, P(GMA) brushes were used for immobilization of invertase and laccase by ionic interactions after hydrazine and amino functionalization, respectively.[11] Sodium 4-styrenesulfonate was grafted on the Fe_3O_4/SiO_2 composite particles by SI-ATRP and used for pectinase immobilization.[12]

Lipases (EC 3.1.1.3) from different sources are currently used in various biochemical reactions including esterification,[13] hydrolysis,[14] aminolysis,[15] and transesterification[16] to produce many useful chemical products. The rising interest in lipase mainly lies on its wide industrial applications, including detergent formulation, oil/fat degradation, pharmaceutical synthesis, cosmetic production, and flavor ester synthesis.[17−19] There are several studies about enzymatic flavor ester synthesis in solvent-free and nonaqueous medium using immobilized lipase. Carboxylic acid esters of low molecular weight are colorless, volatile liquids with pleasant odors. They give flavor and fragrance to fruits and flowers and are used as synthetic flavours and fragrances in the food and cosmetic industries. One of them, ethyl acetate is one of the most important flavor compounds used in the cosmetic and food industries.[20−22] Despite the excellent catalytic properties of lipases, enzyme properties must be improved before their implementation on industrial scale. In general, enzyme reusability and stability can be improved via immobilization techniques. There are many kinds of immobilization techniques,[23−26] among which the multipoint covalent attachment between enzyme molecules and host materials is often accepted as an efficient method to achieve high stability of the enzyme by increasing the rigidity of its structure and reducing protein unfolding. Glutaraldehyde activation of supports is a well-known technique to immobilize enzymes.[26−29] The methodology is quite simple and efficient. In some cases, it will improve enzyme stability by multipoint immobilization.[27−29] It has been shown by Monsan[29] that it is relatively simple to activate aminated supports with glutaraldehyde, having between one or two molecules of glutaraldehyde per primary amino groups. Similar observation was also reported by Betancor et al., at low glutaraldehyde concentration (such as 0.5% glutaraldehyde, (v/v)) only one molecule of glutaraldehyde can be introduced per primary amino group on the support.[28]

* To whom correspondence should be addressed.
[†] Istanbul Technical University.
[‡] Gazi University.

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In this work, P(S-DVB) microspheres were produced by suspension polymerization. A hydrophobic polystyrene block spacer chain on the microsphere surface was created via the SI-ATRP technique. Then a second P(GMA) block as reactive group was introduced via SI-ATRP from terminal bromoalkyl groups of the PS grafts. The covalent immobilization of lipase via diblock grafts on the microspheres was investigated with two different approaches: first, lipase was immobilized by direct binding to the reactive epoxy groups on the polymer brushes with the concomitant formation of hydroxyl groups; second, after attachment of amino functional spacer-arm, the enzyme was immobilized via glutaraldehyde coupling. Lipases have a higher level of hydrophobicity than that of conventional proteins, and the total percentage of hydrophobic amino acids (i.e., Ile, Leu, Val, Met, Tyr, Phe) in lipases isolated from various microbial sources varies between 28 and 33%.[30] Lipases have an inherent active conformation in hydrophobic media (lipases bound on cell membranes and/or in chylomicrometer), and thus a high level of retained lipase activity could be attained by designed nanostructured microspheres possessing hydrophobic and hydrophilic functionalities. In this respect, presence of PS as first block is expected to provide additional hydrophobicity which is useful for the active conformation of the immobilized lipase. The influence of several parameters was investigated and compared, such as activity retention, kinetic properties, and thermal and operational stability aspects. Finally, immobilized lipase was used for banana ester synthesis (i.e., ethyl acetate and isoamyl acetate) either in solvent-free conditions or in nonaqueous medium.

Materials and Methods

Materials. Lipase (from *Rhizomucor miehei* $\geq$ 20 U mg^{-1}; EC 3.1.1.3), hexamethylenediamine (HMDA), glycidylmethacrylate, glutaraldehyde, tributyrin, sodium cholate, CuBr, dibenzoyl peroxide, and gum arabic were supplied by the Sigma-Aldrich Chemical Co. (St. Louis, MO) and used as received. Styrene and divinylbenzene (DVB) were obtained from Merck AG (Darmstadt, Germany), and inhibitor was rendered by washing with NaOH solution (3 wt % NaOH) prior to use. Dibenzoyl peroxide was recrystallized from ethanol. CuBr was freshly prepared using the procedure as described in the literature.[31] The ATRP ligand, H-TETA (1,1,4,7,10,10-hexakis[hexyl-1,4,7,10-tetraazadecane]), was prepared by alkylation of triethylenetetramine with 1-bromohexane as described previously.[32] *N*,*N*,*N*′,*N*′-Tetraethyldiethylenetriamine (TEDETA) and glycidyl methacrylate were distilled before use. All other chemicals were analytical grade products and were purchased from Merck AG (Darmstadt, Germany).

Synthesis of Cross-Linked Microspheres Core P(S-DVB). P(S-DVB) microspheres were prepared by cross-linking copolymerization of the styrene−divinylbenzene mixture (with 9:1 M ratio) in aqueous suspension using gum arabic as stabilizer, according to the literature method.[33] The microspheres were dried and sieved, and the 210−422 μm size fraction was used in further reactions.

Acetoxy Mercuration of P(S-DVB) Microspheres. In a 250 mL three-necked round-bottom flask equipped with reflux condenser, 21 g of PS-DVB (210−422 μm) microspheres was wetted with 50 mL of dry acetic acid and then 10 g of HgO, 40 mL of acetic acid, and 20 mL of acetic anhydride were added to the reaction mixture. The reaction content was refluxed for 3 h at 120 °C, and the resulting mixture was poured into a large excess of water (1.0 L). The microspheres were collected by filtration and washed with excess of water and alcohol (50 mL × 2). Air-dried (the product was not dried in vacuum to avoid sublimation of the mercury) microsphere product weighed 27 g (28.6 wt % mass increase). These results revealed that a maximum of 12% of the styrene units (in mol mol^{-1}) were acetoxymercurated.

Chlorine Exchange Reaction. In a 500 mL flask, mercurated microspheres (27 g) were mixed with 200 mL of saturated NaCl solution and shaken for 24 h at room temperature. Then the reaction mixture was filtered, and the collected microspheres were washed with excess of water and alcohol (30 mL × 2) and dried under open atmosphere at room temperature for 24 h. The dry yield of the chloromercurated product was 26.2 g.

Reaction with 2-Bromoacetyl Bromide. The chloromercurated microspheres (25 g) were mixed with 50 mL of dioxane in a 250 mL flask, and the mixture was shaken for 30 min in a continuous shaker. Then 25 mL (123.8 mmol) of 2-bromoacetyl bromide was introduced to the reaction mixture, and shaking was continued for 24 h at room temperature. The reaction mixture was poured into ice−water, and the microspheres were filtered off. The solid residue was washed with (40 mL × 2) methanol to remove alcohol-soluble mercury bromide and filtered. The dried product (Scheme 1; step-1) microspheres weighed 21.4 g.

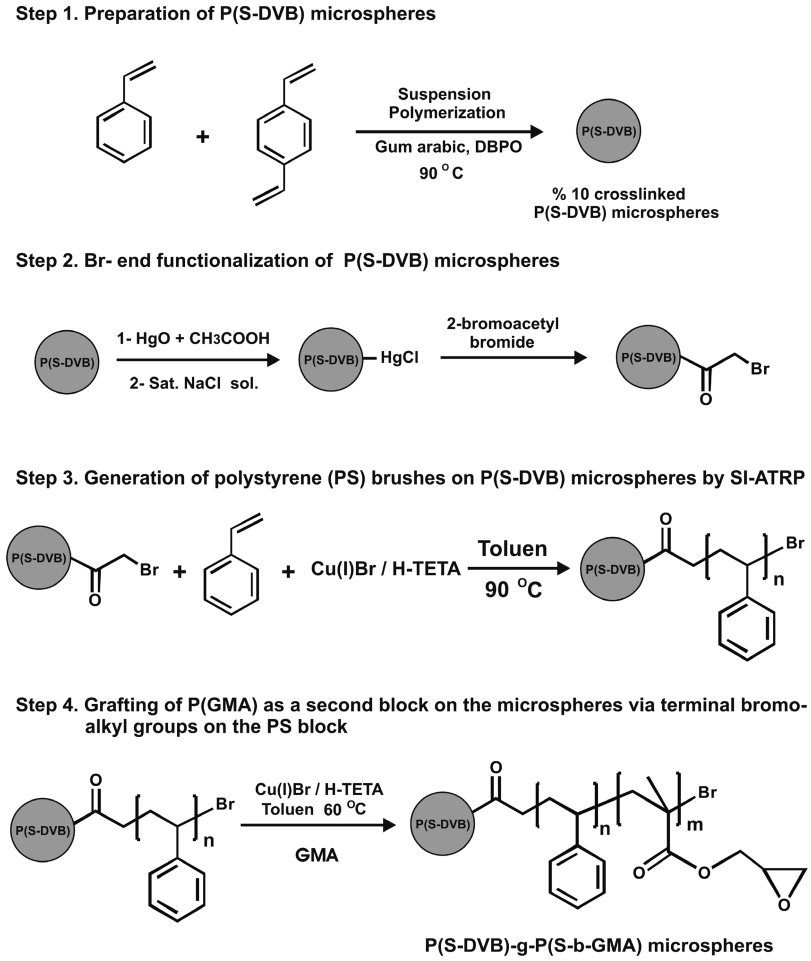
Determination of the Bromine Content. The bromide content of the step-1 microspheres was determined by simple titration. For this purpose, 0.2 g of the bromoacetylated polymer was mixed with 10 mL of methanolic NaOH solution (5 M) in a 50 mL flask and refluxed for 4 h. The mixture was filtered and washed with distilled water, and the filtrate was transferred into a volumetric flask and diluted to 250 mL with distilled water. The NaBr content of the solution was assayed colorimetrically by the mercuric thiocyanate method.[34] This analysis gave 0.84 mmol bromine per gram of the polymer.

Graft Copolymerization of Styrene from the Supported P(S-DVB) Microsphere Core. In a 100-mL three-necked round-bottom flask fitted with nitrogen inlet, 5 g (4.2 mmol) of bromoacetyl functional microsphere product was swelled in 10 mL of dry toluene, and then 30 mL of styrene (0.262 mol), 2.73 g (4.2 mmol) of H-TETA, and 0.6 g of CuBr (4.2 mmol) were added to the reaction mixture under nitrogen atmosphere. The reaction was conducted at constant temperature (90 °C) under continuous stirring for 6 h. The reaction mixture was then cooled, diluted with THF, and filtered. Vacuum dried (at 60 °C for 24 h) product (Scheme 1, step-2) weighed 12.5 g.

Incorporation of Poly(GMA) as the Second Block onto Step 2 Microspheres. To a 100 mL three-necked round-bottom flask equipped with a nitrogen inlet and a reflux condenser were added 30 mL of toluene and 6 mL of GMA (45 mmol). Then 10 g of the step-2 microspheres (with 3.36 mmol active chain ends) was introduced to the flask and left in contact for 1.0 h to swell. To this mixture were added 2.18 g (3.36 mmol) H-TETA and 0.482 g CuBr (3.36 mmol) under nitrogen atmosphere. The reaction was continued for 4.0 h at 60 °C under nitrogen atmosphere. The microspheres were isolated from the mixture by filtering and washed with THF (4 × 50 mL) to eliminate residuals. The product was dried under vacuum at room temperature for 24 h. The yield of the resulting material (step-3) was 12.02 g.

Amino Functionalization of Grafted P(S-SVB) Microspheres. The epoxy groups carrying P(S-DVB)-*g*-P(S-GMA) microspheres were aminated with 0.5 M 1,6-diaminohexane solution (i.e., spacer-arm) at pH 10.0 and at 65 °C in a reactor containing 10 g of microspheres and was stirred magnetically for 5 h. After the reaction, the spacer-arm-attached P(S-DVB)-

Scheme 1. Schematic Representation of the Synthesis and the Chemistry Used for the Grafting of P(S-DVB) via SI-ATRP



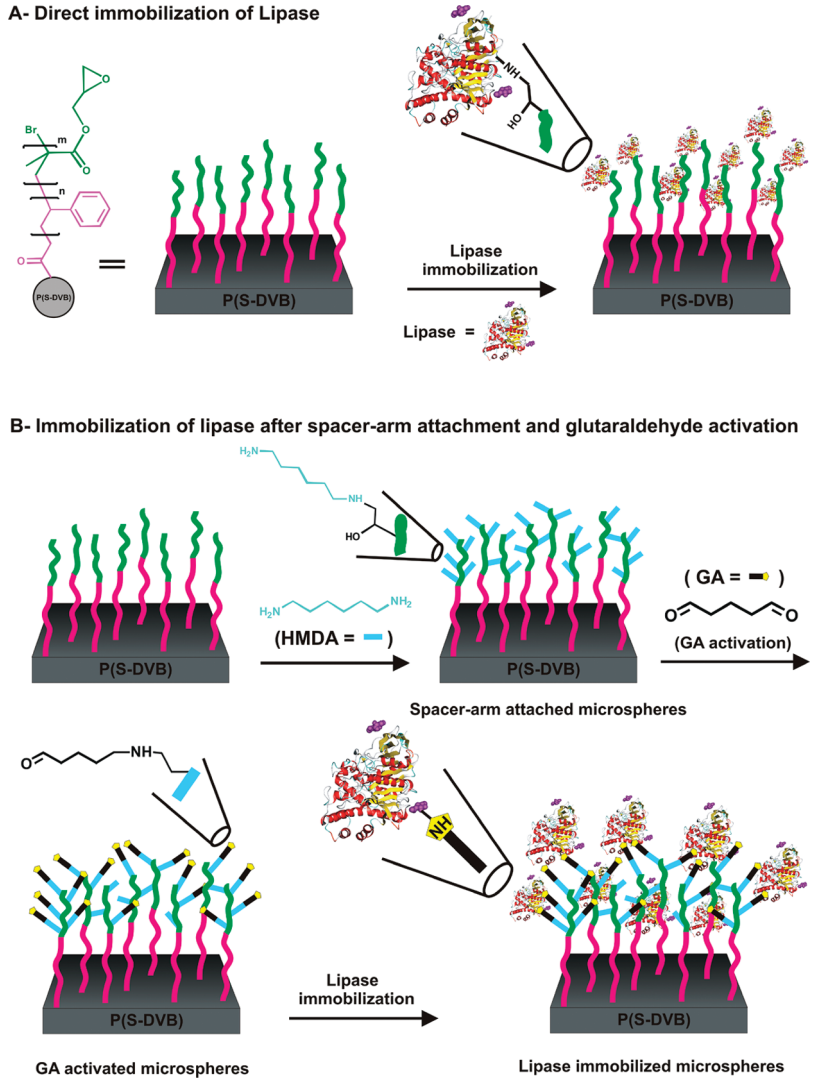
g-P(S-GMA) microspheres were washed with distilled water. The microspheres were equilibrated in phosphate buffer (20 mL, 50 mM, pH 7.0) for 6 h and transferred to the same fresh medium containing GA (20 mL, 0.5 vol % GA). The activation reaction was carried out at 25 °C for 12 h while continuously stirring the medium. After the reaction period, the microspheres were cleaned by washing sequentially with distilled water, acetic acid solution (0.1 M, 100 mL), and phosphate buffer (0.1 M, pH 7.0). The resulting microspheres were used for the immobilization of lipase.

Immobilization of Lipase on Microspheres. P(S-DVB)-*g*-P(S-GMA) and P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres (5.0 g) were equilibrated in phosphate buffer (50 mM, pH 7.0) for 2 h and transferred to the same fresh medium containing lipase (100 mL, 2 mg mL^{-1}). Immobilizations of *Rhizomucor miehei* lipase on both microspheres were carried out at 4 °C for 3 to 18 h while continuously stirring the reaction medium. After this period, the enzyme-immobilized microspheres were immediately transferred ethylenediamine solution (5.0 mg mL^{-1} ethylenediamine) in the same buffer solution to block the free reactive groups on both microspheres. Schematic representation of chemical route for the enzyme immobilization is presented in Scheme 2. Physically bound enzyme from the microspheres was removed by washing with a solution containing ionic detergent (sodium cholate, 50 mM) and salt (NaCl, 1.0 M) at pH 8.0 for 2 h. The amount of immobilized lipase on the P(S-DVB)-*g*-P(S-GMA) and P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres were determined by measuring the initial and final concentrations of protein within the immobilization medium and in wash solutions using Coomassie Brilliant Blue as described by Bradford.[35] A calibration curve constructed with lipase solution of known concentration (0.05−0.50 mg mL^{-1}) was used in the calculation of protein in the enzyme and in wash solutions.

Activity Assays of Lipase. The activity of free and immobilized lipase was determined by olive oil hydrolysis.[36] A 100 mL amount of olive oil emulsion was prepared by mixing olive oil (50 mL) and gum arabic solution (50 mL, 7 wt %, w/v). The assay mixture consisted of emulsion (5 mL), phosphate buffer (2.0 mL, 100 mM, pH 7.5), and free enzyme (0.5 mL, 1.0 mg mL^{-1}) or immobilized enzyme (0.1 g of microspheres). Oil hydrolysis was carried out at 35 °C for 30 min in a shaking water-bath at 150 rpm. The reaction was stopped by the addition of 10 mL of acetone−ethanol solution (1:1, v/v). The liberated fatty acid in the medium was determined by titration with 50 mM NaOH solution. These activity assays were carried out over the pH range 4.0−9.0 and temperature range 20−60 °C to determine the pH and temperature profiles for the free and the immobilized enzymes.

Determination of the Kinetic Parameters of the Enzyme Preparations. The kinetic constants were estimated by the reaction of the free and immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres with tributyrin as substrate (5−50 mM) and titrating the reaction product, butyric acid, with 50 mM NaOH as described above. The experiments were conducted under the optimized assay conditions. The apparent K_m and V_{max} values for the free and immobilized lipase were calculated from Lineweaver−Burk plots by using the initial rates of the enzymatic reaction:

Scheme 2. Chemistry Used for the Covalent Immobilization of Lipase on the P(S-DVB)-*g*-P(S-GMA) and P(S-DVB)-*g*-P(S-GMA)-HMDA Microspheres



$$v^{-1} = \{K_m/V_{max}[S]^{-1}\} + v_{max}^{-1}$$

where [S] is concentration of the substrate, and v and V_{max} represent the initial and maximum rate of reaction, respectively. K_m is the Michaelis constant. One lipase unit corresponded to release of 1 μmol of fatty acid per minute under assay conditions. The specific activity is the number of lipase units per milligram of protein.

Thermal and Storage Stability of the Immobilized Lipases. The thermal stabilities of the free and immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres were determined by incubation in substrate-free phosphate buffer solution (50 mM, pH 7.5) at two different temperatures (55 and 65 °C) under continuous shaking at 150 rpm. At 15 min time intervals, the remaining activities of the free and immobilized lipase were measured as described above.

Enzymatic Synthesis of Banana Flavor Esters. Industrially important esters (i.e., ethyl acetate and isoamyl acetate; banana flavor esters) were synthesized in screw-capped flasks (20 mL) using immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres (0.1 g) for both solvent-free medium and nonaqueous medium. For the solvent-free system, the enzymatic esterification reactions were carried out with various acetic acid/ethyl alcohol and/or acetic acid/isoamyl alcohol molar ratios (i.e., 0.25:1.0, 0.5:1.0, 0.75:1.0, and 1.0:1.0) containing of 20 μL of distilled water) on an orbital shaker at 150 rpm and at 40 and 55 °C, respectively, for 24 h. For nonaqueous medium, ethyl acetate and isoamyl acetate synthesis reactions were carried out in *n*-hexane (15 mL) containing 50 mmol of acetic acid and 100 mM ethyl alcohol and/or 50 mmol of acetic acid and 100 mM isoamyl alcohol, with shaking at 150 rpm at 40 and 55 °C, respectively. During the 24 h reaction, the amount of remaining acid was determined by titration using 50 mM NaOH and phenolphthalein as an indicator. The conversion (%) of esters was calculated based on the conversion of the acetic acid to esters after a given time. The initial reaction rate was determined in the linear region. All of the experiments were carried out in triplicate. The control experiments (no enzyme) showed an ester yield of less than 2%.

Operational Stability of Immobilized Lipase in Ester Synthesis. A batchwise fashion was adopted to investigate the operational stability of the immobilized lipase in ester synthesis using acetic acid/ethyl alcohol and/or acetic acid/isoamyl alcohol molar ratios 0.5:1.0. The activity was determined as described above, and each reaction was terminated after a 12 h reaction period. After any run, the same enzyme immobilized on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres was washed two times with 5.0 mL of ethyl acetate or isoamyl acetate and

reintroduced into a fresh medium, this being repeated up to 10 cycles for ester synthesis.

Characterization of Microspheres. The epoxy group content of the P(S-DVB)-*g*-P(S-GMA) microspheres was determined using a pyridine·HCl method.[36] The available amino group content of the 1,6-diaminohexane-attached microspheres was determined as follows: a 1.0 g amount of microspheres was soaked in water (15 mL) for 24 h. Then 1.0 M HCl (15 mL) was added to the mixture and shaken overnight. At the end of this period, the microspheres were filtered and assayed by titration with 0.1 M NaOH solution in the presence of phenolphthalein. The specific surface area of the P(S-DVB)-*g*-P(S-GMA) microspheres was measured by a surface area apparatus and calculated using the BET (Brunauer, Emmett, and Teller) method. The density of P(S-DVB)-*g*-P(S-GMA) microspheres was determined by using a stereopycnometer (Quantachrome, SPY 3, Syosset, New York).

The Fourier transform infrared (FTIR) spectra were measured on a spectrophotometer (Shimadzu, FTIR 8000, Japan) at room temperature, and the samples were prepared in pellet form using spectroscopic grade KBr. The P(S-DVB), P(S-DVB)-*g*-PS, and P(S-DVB)-*g*-P(S-GMA) microspheres (0.01 g) and KBr (0.1 g) were thoroughly mixed, mixture was pressed to form a tablet, and the spectrum was recorded. The surface morphology of the P(S-DVB)-*g*-P(S-GMA) microspheres was observed by scanning electron microscopy (SEM). The dried microspheres were coated with gold under reduced pressure, and their electron micrographs were obtained using a scanning electron microscope (JEOL, JSM 5600, Japan).

Results and Discussion

Properties of the Functionalized Microspheres. The crucial task in surface-initiated ATRP is generation of the initiation sites on the solid surface. This can be carried out either by surface modification with suitable reagents possessing haloalkyl functionality or by preparation of the solid support using an appropriate functional comonomer. The linkage between solid core and initiator group is also important. Nonhydrolyzibility of this linkage is preferable when the resulting functional material is to be processed under harsh conditions. Attachment of the initiator groups by ester linkage is a common approach and has been widely employed by many authors.[37,38] However, hydrolysis of this bond chemically or enzymatically deters the use of this approach when the hairy carrier polymer will be recycled and reused.

Taking the properties into consideration, in this work, the initiator group was introduced to the solid core in an alternative way in which the chloromercurated surface of the P(S-DVB) microspheres was reacted with bromoacetyl bromide. The resulting bromoacetyl group was utilized as the initiating point. Since the initiator group is being connected to the support and graft chains by C−C linkages, any leakage of graft chains from the support is avoided. This makes the material recyclable and reusable. The length of the graft chain is also important to increase mobility of the graft chains and the rate of the reactions as well. The reaction rates of the functional groups increase as the distance from the bead core increases, as reported by many authors, and this has been termed as the "spacer chain effect". Regarding the spacer chain, PS was first grafted to the P(S-DVB) resin and then P(GMA) was introduced via ATRP from the terminal bromoalkyl groups of the PS grafts. The chemical transformations in each step were followed by monitoring the characteristic bands in the FT-IR spectra of the products (Figure 1). In the spectrum of P(S-DVB-Br) (the product of step 1;

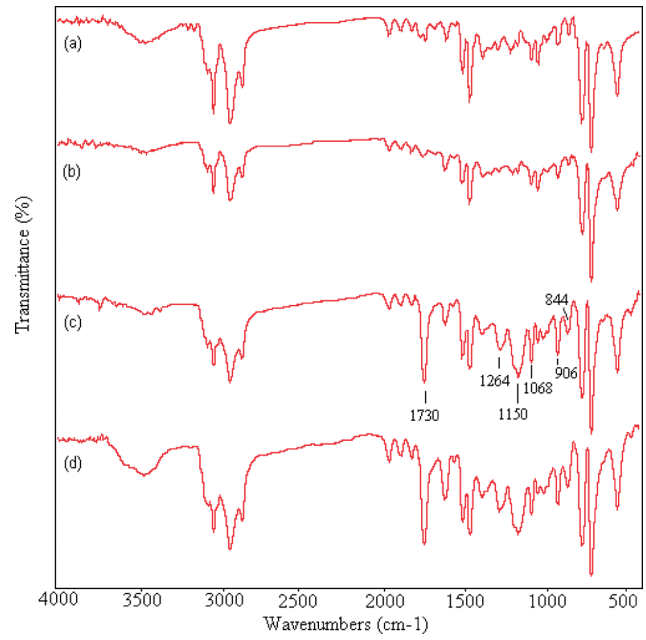


Figure 1. (a) P(S-DVB)-Br (the product from step 1; P(S-DVB)-Br); (b) polystyrene grafted (S-DVB) (the product from step 2; P(S-DVB-*g*-(PS)-Br)); (c) the second block P(GMA) grafted onto the first styrene block (the product from step 3; P(S-DVB)-*g*-P(S-GMA)); (d) spacer-arm-attached P(S-DVB)-*g*-P(S-GMA)-HMDA.

Figure 1a) bearing bromoacetyl groups, the typical stretching vibration band of the carbonyl (C═O) group is observed at 1719 cm^{-1} as a weak band. Apparently, the low intensity of this band is due to the low density of this group in the structure. Also, a very weak band associated with C−Br vibration is observable at 795 cm^{-1}. After grafting with PS (i.e., P(S-DVB)-*g*-PS; the product of step 2; Figure 1b), the C−Br vibration becomes almost invisible which can be ascribed to its consumption by utilization in the grafting with styrene. However, the carbonyl band vibration at 1719 cm^{-1} also becomes invisible, due to the decreasing percentage of this group. After additional grafting with GMA, typical methacrylate ester vibrations are observed at 1725, 1255, and 1150 cm^{-1} (which are associated with stretching vibrations of carbonyl, CO−O, and COO−C bonds, respectively) (the product of step 3; Figure 1c). The typical bands arising from the epoxy functionality are not discernible around 1000 cm^{-1}, because this region is overcrowded with many bands. Nevertheless, the shoulder around 1170 cm^{-1} can be ascribed to the C−O−C stretching vibration of the epoxy group. However, decreasing intensity of this band after modification with the amino compound implies that this band comprises also the vibration of glycidylic ether group. Another change is observed in the 3100−3500 cm^{-1} range which can be considered as the O−H vibration band arising from the ring-opening of the epoxy functionality (Figure 1d). The FTIR spectra of P(S-DVB)-*g*-P(S-GMA)-HMDA also have the characteristic N−H amine stretching bands between 3500 and 3300 cm^{-1} (the bands more broadened and expanded after attachment of 1,6-diaminohexane) and at 1600 cm^{-1}.

The scanning electron microscopy (SEM) micrograph presented in Figure 2 shows a smooth surface structure of the P(S-DVB)-*g*-P(S-GMA)-HMDA microspheres. The nonporous surface properties of the microspheres would reduce the diffusion limitation of the substrate and product during enzymatic hydrolysis and esterification reactions.

The specific surface area of the P(S-DVB)-*g*-P(S-GMA) microspheres was measured with the BET method and was 12.3

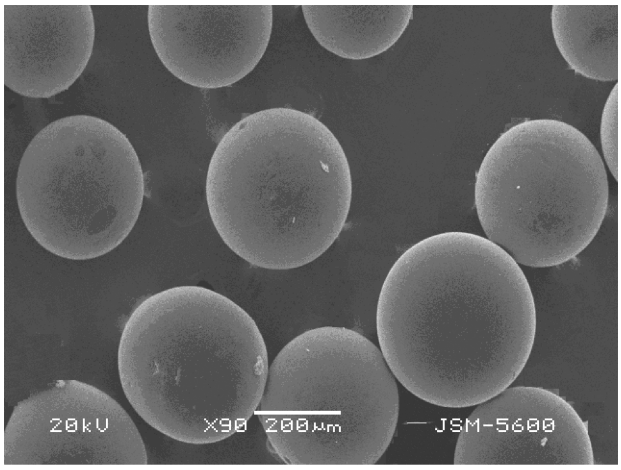


Figure 2. Scanning electron micrograph images of P(S-DVB)-*g*-P(S-GMA) microspheres.

m^2 g^{-1} microspheres. The amount of epoxy groups on the P(S-DVB)-*g*-P(S-GMA) microspheres was determined to be 0.18 mmol g^{-1} microspheres. The amino group content of the P(S-DVB)-*g*-P(S-GMA)-HMDA microspheres was 0.32 mmol g^{-1} microspheres, respectively.

Immobilization of Lipase on the P(S-DVB)-*g*-P(S-GMA)-Based Microspheres. Polymer brushes offer certain advantages over other materials, as they are covalently anchored to the substrate, providing excellent mechanical stability and presenting a high surface area template with functionality controllable by monomer type and brush length. The epoxy groups of P(GMA) coated onto styrene-grafted P(S-DVB)-*g*-P(S) microspheres (i.e., P(S-DVB)-*g*-P(S-GMA)) were converted into amino groups by the reaction with 1,6-diaminohexane, P(S-DVB)-*g*-P(S-GMA)-HMDA. The aim was to determine an efficient relationship between enzyme and support by moving the enzyme away from the surface of the microspheres, via incorporation of spacer arms. Lipase was then covalently immobilized on both P(S-DVB)-*g*-P(S-GMA) and spacer-arm-attached and glutaraldehyde-activated P(S-DVB)-*g*-P(S-GMA)-HMDA microspheres.

In the direct method (Scheme 1a), lipase was immobilized on the P(S-DVB)-*g*-P(S-GMA) microspheres via a coupling reaction between the free amino groups of enzyme and epoxy groups of the support. In the case of P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres (Scheme 1b), the coupling reaction between the amine groups of the enzyme and the aldehyde groups of the supports could be considered via shift base formation. Glutaraldehyde can readily react with amino groups; therefore, the aldehyde group content should be close to the amino group content on the microspheres. The SH groups of lipase can also react with glutaraldehyde after the amine groups have been used. It is important to note that NH_2 groups are more liable than SH groups to react with glutaric dialdehyde. Most proteins contain many lysine residues, usually located on the protein surface (i.e., exposed to the aqueous medium) because of the polarity of the amine group. In addition, lysine residues are generally not involved in the catalytic site, which allows moderate level covalent linkage to preserve protein conformation and thus biological activity.[39] In addition, the attachment of a spacer arm (i.e., 1,6-diaminohexane) to the P(S-DVB)-*g*-P(S-GMA) microsphere surface could prevent undesirable side interactions between large enzyme molecules and support. In this way, more areas for the immobilized lipase could become accessible to lipid substrate. The attachment of the spacer arm resulted in an increase in the apparent activity of the immobilized lipase with respect to the enzyme immobilized via the direct epoxy group coupling. The maximum lipase immobilization capacities of the P(S-DVB)-*g*-P(S-GMA) and P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres were 19.3 and 27.6 mg g^{-1}, respectively. The retained activity yield of the lipase immobilized on the spacer-arm-attached microspheres was about 84.8%, and it was 62.1% for the enzyme immobilized through epoxy groups. The measured specific activity of the free lipase was 21.3 U mg^{-1} protein. The specific activity of lipase immobilized on P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres was 498 U g^{-1} microspheres. Without the spacer arm, the lipase, in attempting to maximize its contact with the surface, could lose its active conformation, and consequently, low specific activity (255 U g^{-1} microspheres) results. The spacer-arm-attached P(S-DVB)-*g*-P(S-GMA)-HMDA microspheres themselves possessed good enzyme immobilization capacity at 27.6 mg protein g^{-1} microspheres. However, the relative amount of the immobilized lipase on the spacer-arm-attached and glutaraldehyde-activated support was about 22.7% higher than that of the lipase immobilized directly on the P(S-DVB)-*g*-P(S-GMA) via epoxy group coupling. The improvement could be attributed to the physical and chemical changes that occurred as a result of the spacer-arm attachment and glutaraldehyde activation. Considering that the spacer arm and coupling agent (i.e., HMDA and glutaraldehyde, respectively) have six- and five-carbon lengths, a total eleven-carbon spacer length should be active in this immobilization method. Thus, the external hydrophobic amino acid residues on the enzyme molecule could not interact too much with hydrophobic polystyrene surface as compared to direct coupling of lipase on the microsphere surface. By incorporating the eleven-carbon spacer-arm on the P(S-DVB)-*g*-P(S-GMA) microspheres, a decrease in the surface hydrophobicity also resulted to further contribute to the improvement in lipase immobilization. All these factors resulted in better immobilization of lipase on the spacer-arm-attached microspheres. In this respect, the polystyrene chain would contribute to a higher hydrophobicity of P(S-DVB)-*g*-P(S-GMA) but the effect would be offset by the amino groups of the spacer arm, which is hydrophilic in nature. As reported previously, excessive hydrophobicity of functional group should also be avoided, as it is reported to be responsible for significant loss of lipase enzymatic activity.[23] Therefore, the rest of the immobilization study was carried out using the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres.

The effects of enzyme coupling time on the immobilization capacity were studied with P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres and are presented in Figure 3. An increase in the coupling duration time led to an increase in the immobilization efficiency (from 9.6 to 27.6 mg g^{-1} microspheres), but this relationship leveled off at around 12.0 h. Further increase in the coupling duration time (up to 18 h) did not lead to a significant change in the immobilization capacity.

Effect of pH and Temperature on the Free and Immobilized Lipase Activity. The effect of pH on the activity of the free and immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA was studied at 40 °C in the pH range between 4.0 and 9.0. The optimum pH of free lipase was 7.5, which corresponds closely to the optimum found for immobilized lipase, which is 8.0 (Figure 4). The pH profile of the immobilized lipase also closely follows that of free lipase. This shift depended on the method of immobilization as well as the structure of the matrix. It should be noted that the shift to the neutral and basic region of the optimal pH range upon immobilization could be expected as a result of the diffusional

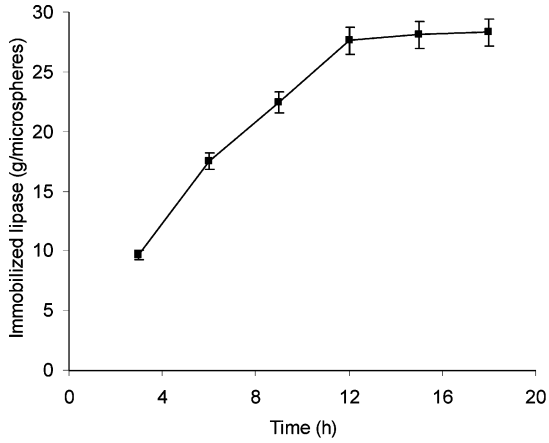


Figure 3. Effect of coupling duration time on the immobilization efficiency of the lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres.

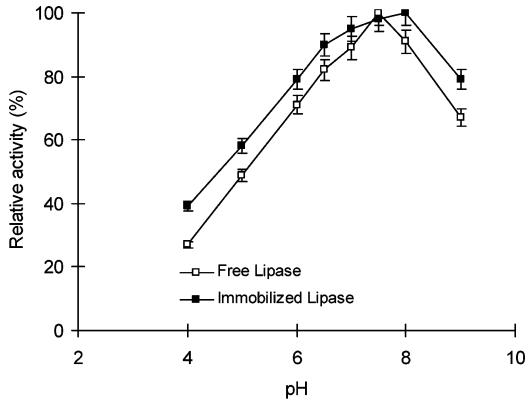


Figure 4. Effect of pH on the activity of free and immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres; the relative activities at the optimum pH were taken as 100% for free and immobilized lipase, respectively.

constraint of the support retaining a higher concentration of enzyme product, fatty acids, on the surface of the microspheres where immobilized lipase is present. It should noted that additional interaction of the support with enzyme due to the presence of amino functional groups on the spacer arm might have also reduced the sensitivity of enzyme to less alkaline pH. This charged group would have better interactions with the hydrophilic sites on the lipase molecule while the hydrophobic sites were still available for catalyzing the esterification reaction. As previously reported, the hydrophobic sites of the lipase molecule are mainly responsible for the catalytic activity.[40−43] The changes in the activity profile of the immobilized enzyme compared to free enzyme could probably be attributed to the stabilization of lipase molecules resulting from multipoint covalent attachment to the surface of the hydrophobic/hydrophilic microspheres, which limited the transition of enzyme conformation against the change of pH. Other researchers have reported similar observations upon immobilization of lipase and other enzymes.[44,45]

The effect of temperature on the free and immobilized enzyme activity was investigated in phosphate buffer (0.1 M, pH 7.5) in the temperature range 20−60 °C. The apparent temperature optimum for free enzyme was about 40 °C while that for the immobilized enzyme and the form stabilized by immobilization was about 45 °C (Figure 5). In the literature, most immobilized lipases exhibited higher optimum temperature values than that of their free counterpart.[46,47] The multipoint covalent attachment of the lipase molecule on the microsphere surface via glutaraldehyde coupling could reduce the conformational flexibility of the enzyme and might result in higher activation energy for the molecule to reorganize to a proper conformation for binding to its large substrate (i.e., olive oil). Thus, the immobilized lipase showed its catalytic activity at a higher reaction temperature compared to that of the free counterpart.

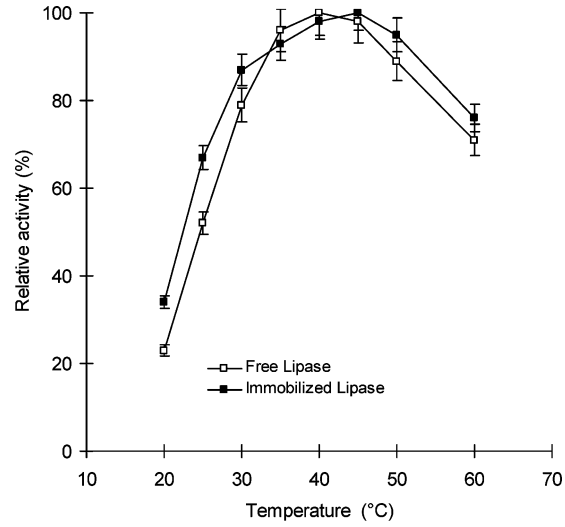


Figure 5. Effect of temperature on the activity of the free and immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres; the relative activities at the optimum temperature were taken as 100% for free and immobilized lipase, respectively.

Kinetic Parameters of the Free and Immobilized Lipases. The K_m value indicates the affinity of an enzyme for a substrate; a lower K_m value represents a higher affinity between enzyme and substrate. In this work, kinetic constants of free and immobilized lipase, i.e. K_m and V_{max} values, were determined by using tributyrin as substrate. The activities of the free and immobilized lipase for various concentrations of the substrate (5−50 mM) were plotted in Lineweaver−Burk plots, and K_m and V_{max} values were calculated from the intercepts on the *x*- and *y*-axis, respectively. The kinetic data for hydrolysis of tributyrin was fitted to the Michaelis−Menten equation. For the free and immobilized lipase, the apparent K_m values were found to be 22.7 and 34.3 mM, respectively. The K_m value of immobilized lipase was the same order of magnitude and was only 1.15 times higher than that of the free enzyme. The V_{max} values for the free and immobilized lipase were calculated as 23.4 and 20.3 U mg^{-1} enzyme, respectively. The V_{max} value of the immobilized enzyme decreased about 1.5-fold compared to the free enzyme. These results indicated that the presented immobilization method changed the reaction velocity and substrate affinity of the *R. miehei* lipase. Several reasons can account for the variations of the V_{max} values of the enzyme upon immobilization.[48,49] These variations are attributed to several factors such as that the covalent attachment of the enzyme molecule on the microsphere surface might have induced an inactive conformation of the enzyme molecules. Furthermore, the immobilization process does not control the proper orientation of the immobilized enzyme on the support. This improper fixation and/or the change in the property of the active sites might hinder the active site for the binding of substrates (i.e., tributyrin) to the immobilized lipase molecules.

The efficiency factor η can be calculated from the maximum reaction rates of the immobilized enzyme over that of the free counterpart:

$$\eta = \upsilon_{\text{immobilized}}/\upsilon_{\text{free}}$$

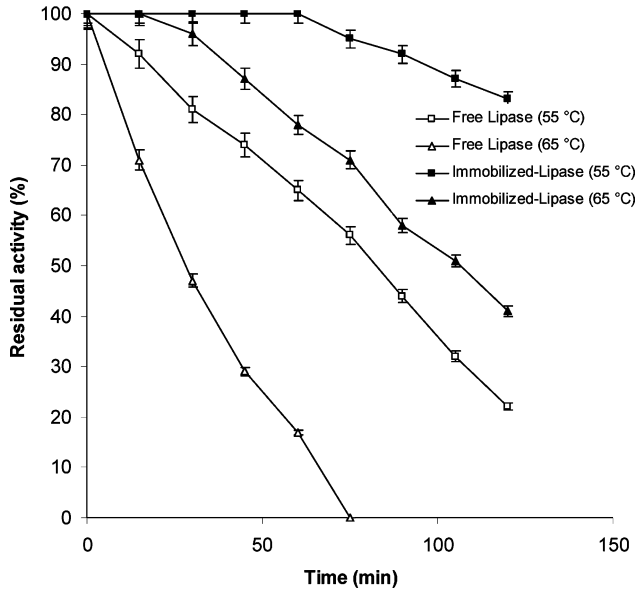


Figure 6. Thermal stability of the free and immobilized lipase at two different temperatures.

where $\upsilon_{immobilized}$ and υ_{free} are the reaction rates of the immobilized and free enzyme, respectively. From this calculation, the nanostructured microsphere−enzyme system provided an efficiency factor of 0.87 for the immobilized lipase. The ratio A_{max}/K_m defines a measure of the catalytic efficiency of an enzyme−substrate pair. In this study, the catalytic efficiencies (A_{max}/K_m) of the free and immobilized lipase were 10.3 and 5.9, respectively. The catalytic efficiency of lipase was decreased about 1.7-fold upon immobilization.

Thermal Stability of the Free and Immobilized Lipase. The effect of temperature on the stability of the free and immobilized lipase is shown in Figure 6. The pattern of heat stability indicated that a smaller rate of thermal inactivation occurred for the immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres than that for the free enzyme. At 55 °C, the free enzyme lost about 78% of its initial activity after 120 min of heat treatment while the immobilized enzyme showed significant resistance to thermal inactivation (lost about 17% of its initial activity in the same period). At 65 °C, the free lipase lost all of its initial activity after 75 min heat treatment. Under the same conditions, the immobilized lipase retained about 41% of its initial activity. As in Figure 6, the free enzyme at 55 and 65 °C exhibited a steep loss of catalytic activity compared to immobilized counterpart, which is typical of most enzymes,[51,52] whereas a marked enhancement of thermal stability was achieved with enzyme immobilization since no activity decay was observed after 60 and 15 min exposure at 55 and 65 °C, respectively. These results suggest that the thermostability of immobilized lipase becomes significantly higher at higher temperature. If the heat stability of enzymes increase upon immobilization, the potential application of such enzymes would be extended.

Enzymatic Activity in Banana Ester Synthesis. The biosynthesis of esters is currently of much commercial interest because of the increasing popularity and demand for natural products among consumers.[43] The activity of immobilized lipase was investigated on the basis of the esterification of ethanol and isoamyl alcohol with acetic acid to produce banana flavors (i.e., ethyl acetate and isoamyl acetate). The enzyme activity was defined based on the percentage of acid conversion. The optimum temperature for the synthesis of ethyl acetate from ethanol and acetic acid by immobilized enzyme, the esterification reaction, was carried out at different temperatures (35−65 °C) and a 1:4 acid/alcohol molar ratio. The effect of temperature on the esterification reaction is presented in Figure 7. For ethyl acetate production, maximum ester synthesis activity was observed at 40 °C with a 1:2 acetic acid/ethyl alcohol molar ratio. On the other hand, the temperature range from 35 to 55 °C, there were remarkable increases in the amount of synthesized isoamyl acetate, and above 55 °C, the ester synthesis yield decreased. Only 7% and 13% conversions were observed at 65 °C for ethyl acetate and isoamyl acetate, respectively. This may be due to the thermal denaturation of enzyme.

The effect of acid to alcohol molar ratio (i.e., acetic acid/ethyl alcohol, and acetic acid/isoamyl alcohol molar ratio (i.e., 0.1:1.0, 0.25:1.0, 0.5:1.0, 0.75:1.0, and 1.0:1.0) at optimal temperatures for each individual ester was investigated using immobilized *Rhizomucor miehei* lipase in a solvent-free system. As seen in Figure 8, the highest amount of ethyl acetate was synthesized (19.7%) with 1:4 acetic acid/ethyl alcohol molar ratio. On the other hand, the amount of synthesized isoamyl acetate increased from 13.4 to 39.7% with increasing acetic acid to isoamyl alcohol molar ratio from 0.1:1.0 to 0.75:1.0 molar ratio. As shown in Figure 8, as the molar ratio of acid/alcohol was further increased, the conversion (%) decreased, and this could be due to the inhibition of the lipase activity by high acetic acid concentration. As previously reported, in the lipase-catalyzed esterification reaction, the first step consists of the preferential binding of the acid molecule to the enzyme molecule.[50] At high alcohol/acid molar ratio, the high alcohol concentration may promote the binding of alcohol molecules to the immobilized lipase, during the first reaction step, competing with the acetic acid. As a result, a decrease in the amount of bound acid occurs. Thus, this situation would lead to a decrease in the reaction rates, since the reaction would be limited by the amount of acid in the vicinity of the enzyme.[24]

The synthesis of ethyl acetate by immobilized *Rhizomucor miehei* lipase was also studied in nonaqueous medium. The ethyl acetate and isoamyl acetate synthesis experiments were carried out under 1:2 acid/alcohol molar ratio for both esterification reactions. The amount of synthesized ethyl acetate and isoamyl alcohol was 34.6 and 59.3% in the presence of n-hexane. The amounts of synthesized ethyl acetate and isoamyl alcohol were increased in the presence of n-hexane compared to solvent-free systems. As reported previously, the presence of an organic solvent can shift the equilibrium toward ester synthesis by a total transfer of ester into the organic phase.[52,53] Similar results related to the incremental increase in the amount of ester synthesized in organic medium were reported previously in the literature. For example, Karra-Chaabouni et al.[54] produced ethyl valerate and hexyl acetate in hexane, heptane, and solvent-free medium. They found remarkably higher ester yields in both hexane and heptane medium than those obtained in the absence of organic solvents.

Operational Stability in Ester Synthesis. In general, enzymes are difficult to recover and reuse. Therefore, the reuse of an immobilized enzyme is of key importance for industrial applications, and an increased stability could make the immobilized enzyme more advantageous than its free counterparts. In this study, the immobilized lipase on P(S-DVB)-*g*-(S-GMA)-HMDA was repeatedly used as the biocatalyst for the esterification reactions and was subsequently recovered and reused. As seen in Figure 9, the activity of immobilized enzyme slightly decreased as the number of reuses increased. The same activity values for isoamyl acetate synthesis were obtained within the

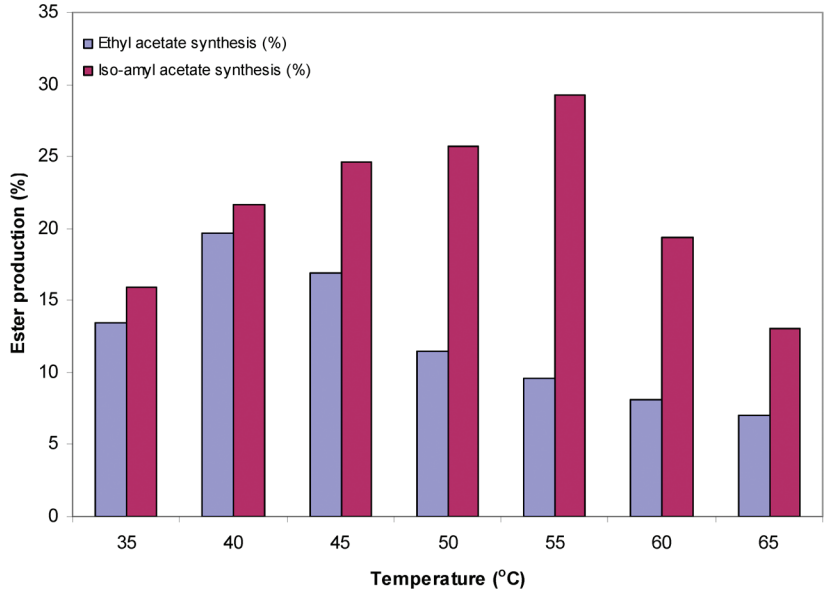


Figure 7. Effect of temperature on the enzymatic production of ethyl acetate and isoamyl acetate using immobilized lipase on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres.

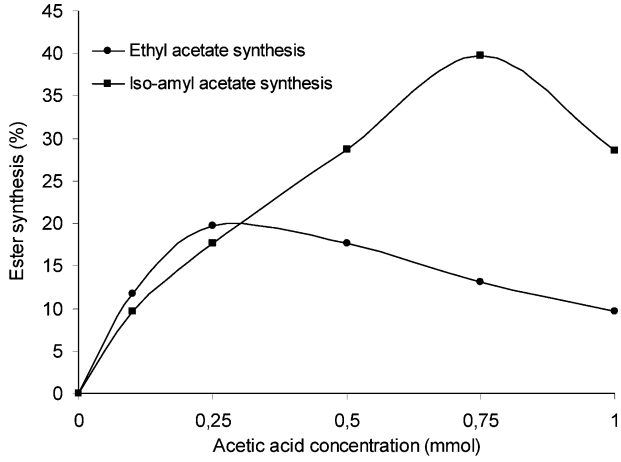


Figure 8. Effect of acid/alcohol ratio on the ester synthesis activity of the immobilized lipase.

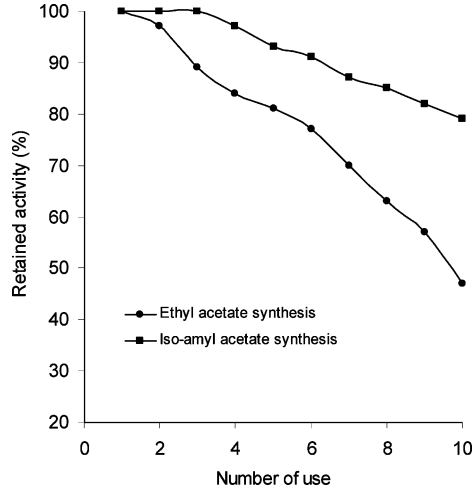


Figure 9. Operational stability of immobilized lipase during ethyl acetate and isoamyl acetate synthesis. The same enzyme microspheres were used for each batch during repeated use.

first three cycles. In this case, the activity retention of the enzyme immobilized for isoamyl acetate synthesis was found to be slightly better than that of the ethyl acetate synthesis. As seen in this figure, immobilized enzyme retained 47% and 79% of its initial activity during synthesis of ethyl acetate and isoamyl alcohol after 10 cycles of use, respectively, possibly resulting from the inactivation of lipase upon use. This result showed that lipase immobilized on the nanostructured microspheres could be used successfully for industrial applications requiring long-term reaction stability. Thus, this immobilization method led to increased enzyme reusability.

Conclusions

In the present study, new hydrophobic/hydrophilic diblock polymers were grafted on P(S-DVB) microspheres using styrene and GMA monomers via the SI-ATRP method. Poly(styrene-*b*-glycidyl methacrylate) diblock brushes grown by ATRP from surface-tethered initiators were examined as a potential surface for the immobilization of lipase. The lipase was covalently immobilized on the microspheres by direct binding of P(S-DVB)-*g*-P(S-GMA) and after spacer-arm attachment and glutaraldehyde coupling (i.e., P(S-DVB)-*g*-P(S-GMA)-HMDA-GA). The results indicate that brushes can bind 19.3 and 27.6 mg g^{-1} lipase with activities of 62.1% and 84.8% relative to those of free enzyme. From these studies, spacer arms can be effectively used to immobilize lipase for an increase in activity of more than 22.7% active compared to that without a spacer arm. The optimum temperature and pH for the immobilized lipase on P(S-DVB)-*g*-P(S-GMA)-HMDA-GA and the thermal stability and operational stability were experimentally studied. The optimum pH values were 7.5 and 8.0 for free and immobilized lipase, respectively. Immobilized *Rhizomucor miehei* lipase on P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres was used in the synthesis of banana flavor esters. After 10 reaction cycles, the residual activities were 47% and 79% of its initial activities for ethyl acetate and isoamyl acetate synthesis, respectively. The immobilized enzyme on the P(S-DVB)-*g*-P(S-GMA)-HMDA-GA microspheres described in this study could effectively be used in enzymatic reactions, notably in aroma ester production processes.

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