Bioresource Technology 102 (2011) 3653–3661

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Immobilization of catalase via adsorption on poly(styrene-*d*-glycidylmethacrylate) grafted and tetraethyldiethylenetriamine ligand attached microbeads

Gulay Bayramoglu [a,*], Bunyamin Karagoz [b], Meltem Yilmaz [c], Niyazi Bicak [b,*], M. Yakup Arica [a]

[a] Gazi University, Faculty of Arts and Sciences, Biochemical Processing and Biomaterial Research Laboratory, Teknik Okullar, 06500 Ankara, Turkey
[b] Istanbul Technical University, Department of Chemistry, Maslak, 34469 Istanbul, Turkey
[c] Gazi University, Institute of Science and Technology, Department of Environmental Sciences, 06570 Ankara, Turkey

ARTICLE INFO

Article history:
Received 27 October 2010
Received in revised form 5 December 2010
Accepted 6 December 2010
Available online 13 December 2010

Keywords:
Catalase
Enzyme immobilization
Adsorption
Multi-modal ligand
Kinetics

ABSTRACT

Fibrous poly(styrene-*d*-glycidylmethacrylate) (P(S-GMA)) brushes were grafted on poly(styrene-divinylbenzene) (P(S-DVB)) beads using surface initiated-atom transfer radical polymerization (SI-ATRP). Tetraethyldiethylenetriamine (TEDETA) ligand was incorporated on P(GMA) block. The multi-modal ligand attached beads were used for reversible immobilization of catalase. The influences of pH, ionic strength and initial catalase concentration on the immobilization capacities of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads have been investigated. Catalase adsorption capacity of P(S-DVB-*g*-P(S-GMA)-TEDETA beads was found to be 40.8 ± 1.7 mg/g beads at pH 6.5 (with an initial catalase concentration 1.0 mg/mL). The K_m value for immobilized catalase on the P(S-DVB-*g*-P(S-GMA)-TEDETA beads (0.43 ± 0.02 mM) was found about 1.7-fold higher than that of free enzyme (0.25 ± 0.03 mM). Optimum operational temperature and pH was increased upon immobilization. The same support was repeatedly used five times for immobilization of catalase after regeneration without significant loss in adsorption capacity or enzyme activity.

1. Introduction

Biotechnological and biomedical applications requiring adhesion, adsorption, biocompatibility, permeability and/or other properties depend on the surface properties of the material significantly (Bhattacharya and Misra, 2004; Arica et al., 2010; Karagoz et al., 2010). The modification of material surface is mostly required to change the character of the base support surface from hydrophobic to hydrophilic and/or negatively or positively charged in order to create selective absorptive surface for the adsorption of protein (Arica et al., 2004; Bayramoglu and Arica, 2009). Among the surface functionalization techniques, fibrous polymer brushes from SI-ATRP have been widely used to tailor the surface properties of materials (Xu et al., 2009; Acar and Bicak, 2003; Cullen et al., 2008; Jain et al., 2010; Bayramoglu et al., 2010). Various monomers with chemical functionalities can be grafted onto the surface. Among them, monomer with the epoxy group is attractive because it can be readily modified using various chemical reactions to introduce various functional moieties (Bıcak et al., 2006; Arica and Bayramoglu, 2004; Arica et al., 2009, 2007; Sun et al., 2006). Thus, epoxy groups carrying polymers can be subjected to numerous modification possibilities under mild reaction conditions (Arica and Bayramoglu, 2004; Yavuz et al., 2009).

Many protocols for enzyme immobilization involve irreversible binding to a functionalized support. In the reversible enzyme immobilization, the supports could be regenerated using a suitable desorption agent, and they be recharged again with a fresh enzyme. On the other hand, when the covalently immobilized enzyme becomes inactivated upon use both the enzyme and the support should be eliminated as wastes (Bayramoglu et al., 2010a, 2008; Zhou, 2010). The reversible immobilization of enzyme on the ligated support is based on the non-covalent interactions between enzyme and support. In the case of multi-modal ligand, such interactions increase by electrostatic forces and hydrogen bonding in addition to hydrophobic interactions (Arica and Bayramoglu, 2006; Wang et al., 2010; Bolivar et al., 2009; Torres et al., 2005). The target protein itself is also a multimodal molecule. A fibrous polymer grafted and multimodal ligand incorporated polymeric beads, could be suitable for reversible immobilization of enzyme due to their intrinsically high specific surfaces, providing the quantity and accessibility of the electrostatic and hydrophobic binding sites necessary for high immobilization capacity and large surface area for enzymatic reaction (Arica et al., 2010; Cheng et al., 2010; Lei et al., 2009; Cullen et al., 2008; Sun et al., 2006). The multi-modal ligands on the polymer brushes can interact through the electrostatic and hydrophobic binding sites of the acidic protein around their isoelectric points. Thus,

* Corresponding authors. Tel.: +90 312 202 1142 (G. Bayramoglu), +90 212 285 3221 (N. Bicak).
E-mail addresses: g_bayramoglu@hotmail.com (G. Bayramoglu), bicak@itu.edu.tr (N. Bicak).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.12.029

Nomenclature

BET	Brunauer, Emmett and Teller method
CAT	catalase
C_i	concentrations of the catalase in the initial solution (mg/mL)
C_t	concentrations of the catalase in the supernatant after adsorption (mg/mL)
H-TETA	1,1,4,7,10,10-hexakis(hexyl-1,4,7,10-tetraazadecane)
K_F	adsorption capacity (Freundlich constants)
K_m	the Michaelis constant (mM)
K_T	equilibrium binding constant corresponding to the maximum binding energy (mL/mg)
n	adsorption intensity (Freundlich constants)
P(S-DVB)	poly(styrene-divinylbenzene)
P(S-DVB)-*g*-P(S-GMA)	poly(styrene-divinylbenzene)-*graft*-poly(styrene-glycidylmethacrylate)
P(S-GMA)	poly(styrene-glycidylmethacrylate)
q	amount of catalase adsorbed on the beads (mg/g)
q_T	differential surface capacity for protein adsorption per unit binding energy (mg protein/g beads)
SI-ATRP	surface-initiated atom transfer radical polymerization
TEDETA	*N*,*N*,*N*′,*N*′-tetraethyldiethylenetriamine (multimodal ligand)
THF	tetrahydrofuran
V	volume of the aqueous phase (mL)
v_{free}	reaction rate of the free enzyme
$v_{immobilized}$	reaction rate of the immobilized
V_{max}	maximum rate of reaction (U/mg proteins)
w	weight of P(S-DVB)-*g*-P(S-GMA)-TEDETA beads (g)
ΔG_{max}	maximum binding energy (kJ/mol)
η	efficiency factor

enzyme immobilization onto micro-beads via adsorption by the multi-modal ligand could also provide strong electrostatic and hydrophobic interaction between the enzyme and support (Yavuz et al., 2009; Yang et al., 2007). The multi-dentate ligand could hold certain advantages as a multi-modal ligand for various large-scale industrial applications (Johansson et al., 2003; Chang et al., 2007). For example, these molecules are resistant to harsh chemicals, temperature and high salt concentrations. In addition, this kind of support can be recycled after inactivation of immobilized enzyme and offers cost effective processes (Bayramoglu et al., 2008; Torres et al., 2005).

Catalase is a heme-containing metallo-enzyme that is regarded as one of the most common enzymes in plant and animal tissues and has a protection function related to the decomposition of hydrogen peroxide. Catalase consists of four subunits, each of which includes ferri-porphyrin as a prosthetic group (Boland et al., 1989). Immobilized catalase has useful applications in the food industry in the removal of excess hydrogen peroxide from food products after cold pasteurization and in the analytical field as a component of hydrogen peroxide and glucose biosensor systems (Jurgen-Lohmann and Legge, 2006). Catalase also improves the flavor and/or flavor stability of the finished beer by removal of formed hydrogen peroxide during beer fermentation process.

In the present study, P(S-DVB) beads were prepared by suspension polymerization and modified with poly(styrene-*b*-glycidylmethacrylate) brushes using surface-initiated atom transfer radical polymerization aiming to decorate the beads surface with a fibrous polymer. A multi-modal ligand (i.e., TEDETA) was incorporated to the p(GMA) block. The immobilization parameters such as adsorption conditions (e.g., initial catalase concentration, pH and ionic strength) were varied to evaluate the nature of binding mechanisms of catalase on the multimodal ligand incorporated beads. Finally, catalytic properties of the resultant adsorbed catalase such as activity retention and reusability were investigated and compared to that of the free enzyme.

2. Methods

2.1. Materials

Catalase (hydrogen peroxide oxidoreductase; EC.1.11.1.6) from *Aspergillus niger* (about 4000 U/mg protein; C 3515), CuBr, dibenzoyl peroxide, tetrahydrofuran (THF) and Gum Arabic were supplied by the Sigma–Aldrich Chem. Co. (St. Louis, MO, USA) and used as received. Styrene and divinyl benzene (DVB) were obtained from Merck AG (Darmstadt, Germany), and inhibitor was rendered by washing with NaOH solution (3%, w/w) prior to use. Dibenzoyl peroxide was recrystallized from ethanol. CuBr was freshly prepared by the procedure as described in the literature (Furniss et al., 1978). The surface-initiated atom transfer radical polymerization (SI-ATRP) ligand (1,1,4,7,10,10-hexakis[hexyl-1,4,7,10-tetraazadecane]) (H-TETA) was prepared by alkylation of triethylenetetramine with 1-bromohexane as described before (Acar and Bicak, 2003). *N*,*N*,*N*′,*N*′-Tetraethyldiethylenetriamine (TEDETA) and glycidyl methacrylate were distilled before use. All other chemicals were of analytical grade products and were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of 2-bromoacetyl bromide modified P(S-DVB) beads

Polystyrene-divinylbenzene (S-DVB) beads were prepared by suspension copolymerization of styrene with divinylbenzene (ratio was 9:1 M) using Gum Arabic as stabilizer as reported earlier (Karagoz et al., 2010). The beads were dried, sieved and 210–422 μm size of fraction was used in further reactions (Supplementary Fig. S1). The P(S-DVB) beads were modified with acetoxy mercuration reaction. It was carried out in a reactor containing 10 g HgO in acetic acid (40 mL) and acetic anhydride (20 mL) solution. The P(S-DVB) beads were transferred (21 g wetted before use with 50 mL of dry acetic acid, and the reaction content was refluxed for 3 h at 120 °C. The resulting reaction mixture was poured in distilled water (1.0 L). The beads were collected by filtration and washed with sequentially excess water and alcohol. After air-drying, the results revealed that maximum 12% of styrene units (in mol mol^{-1}) have been acetoxymercurated on the beads. The acetoxymercurated beads (27 g) were subjected to chlorine exchange reaction in saturated NaCl solution (200 mL) for 24 h at room temperature. After this reaction, the beads were washed sequentially with excess of water and alcohol, and then dried. Finally, the beads were then reacted with 2-bromoacetyl bromide. For this reaction, the chloromercurated beads (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 (or 123.8 mmol) 2-bromoacetyl bromide was introduced to the reaction mixture and shaking was continued for 24 h at room temperature. The reaction mixture was transferred in ice waterbath and was filtered. The beads were washed with excess methanol to remove alcohol-soluble mercury bromide. (Supplementary Fig. S1, Step 2).

2.3. Grafting of P(S-DVB) beads with poly(styrene) and poly(GMA)

Grafting of 2-bromoacetyl bromide modified P(S-DVB) beads with poly(styrene) and poly(GMA) were carried out in a glass

reactor. The 2-bromoacetyl bromide modified P(S-DVB) beads (5 g, 0.84 mmol/g) were charged to the reactor and swelled in dry toluene (10 mL) for 2 h. After this period, styrene (30 mL or 0.262 mol), H-TETA (2.73 g or 4.2 mmol) and CuBr (0.6 g or 4.2 mmol) were transferred into the reactor under nitrogen atmosphere. The grafting reaction was carried out at 90 °C for 6.0 h under continuous stirring. The reaction mixture was transferred in THF. The beads were filtered and dried under reduced pressure at 60 °C for 24 h, (Supplementary Fig. S1, Step 3).

The P(GMA) was also grafted as a functional second block on the poly(styrene)-*g*-P(S-DVB) beads using SI-ATRP method. For this grafting procedure, the poly(styrene)-*g*-P(S-DVB) beads (10 g, with 3.36 mmol active chain ends), toluene (30 mL) and GMA (6 mL or 45 mmol) were transferred in a three-necked round bottom flask (with a nitrogen inlet and a reflux condenser). The medium left in contact for 1.0 h to swell. Then, H-TETA (2.18 g or 3.36 mmol) and CuBr (0.482 g or 3.36 mmol) were added under nitrogen atmosphere. The grafting reaction was carried out at 60 °C for 4.0 h under continuous stirring. The beads were collected by filtering and washed with THF to eliminate residuals. The product was dried under reduced pressure at room temperature for 24 h. The yield of the resulting material was 12.02 g (Supplementary Fig. S1, Step 4).

2.4. Incorporation of multimodal N,N,N′,N′-tetraethyldiethylenetriamine ligand via P(GMA) block

To a 100 mL volume of two-necked round bottom flask equipped with a reflux condenser and a dropping funnel, there was added 5 g of P(GMA) grafted beads and 20 mL THF. Then 5 g of distilled (TEDETA) was added to the mixture. The mixture was stirred for 24 h at room temperature and then heated to 60 °C for 4 h. The beads were collected by suction and washed with excess water. The residual amine impurities, were removed by *Soxhlet* extraction using THF as extracting solvent. The product was dried under vacuum at 45 °C for 24 h and weighted 5.4 g (Supplementary Fig. S1, Step 5).

2.5. Reversible immobilization of catalase on P(S-DVB)-g-P(S-GMA)-TEDETA beads

Catalase immobilization on the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was studied at various pHs, in either acetate (5.0 mL, 50 mM, pH 3.0–5.0) or in phosphate buffer (Na_2HPO_4/KH_2PO_4 buffer system; 5.0 mL, 50 mM, pH 6.0–8.0). The initial catalase concentration was 1.0 mg/mL (or 5 mg catalase) in the corresponding buffer. The effects of ionic strength on catalase immobilization via adsorption was carried out in phosphate buffer (50 mM, pH 6.5) for the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads at different NaCl concentrations (between 0.0 and 1.5 M). The maximum catalase immobilization capacity of P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was determined by varying the catalase concentration in the medium between 0.5 and 3.0 mg/mL (or 2.5 and 15 mg catalase in 5 mL medium) at pH 6.5. The immobilization experiments were conducted at 15 °C while continuously stirring for 2 h. After this period, catalase immobilized beads were removed from the enzyme solution and washed with same buffer three times. It was then stored at 4 °C in fresh buffer until use.

The amount of immobilized catalase was calculated as

$$q = (C_i - C_t)V/w \quad (1)$$

where q is the amount of catalase adsorbed on the beads (mg/g), C_i and C_t are the concentrations of the catalase in the initial solution and in the supernatant after adsorption, respectively (mg/mL), V is the volume of the aqueous phase (mL), and w is the weight of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads (g).

2.6. Determination of immobilization efficiency

The amount of immobilized catalase on the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads were determined by measuring the initial and final concentrations of protein within the immobilization medium and in wash solutions using Coomassie Brilliant Blue. A calibration curve constructed with catalase solution of known concentration (0.02–0.50 mg/mL) was used in the calculation of protein in the enzyme and in wash solutions.

The enzyme leakage from TEDETA ligand attached beads was studied in the different enzyme operation conditions as described above. Any measurable enzyme leakage was not observed under all these studied conditions.

2.7. Activity assays of free and immobilized catalase

Catalase activity was determined spectrophotometrically, by direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme. Hydrogen peroxide solutions (5–30 mM) were used to determine the activity of both the free and immobilized enzyme. A 4.0 mL of reaction mixture was equilibrated at 25 °C, for 10 min, and the reaction was started by adding 50 μL of catalase solution (0.1 mg solid/mL). The decrease in absorbance at 240 nm was recorded for 5 min. The rate of change in the absorbance was calculated from the initial linear portion with the help of the calibration curve (the absorbance of hydrogen peroxide solutions of various concentrations (5–30 mM) at 240 nm). One unit of enzyme activity is defined as the decomposition of 1.0 μmol hydrogen peroxide per min at 25 °C and pH 7.0. Catalase immobilized P(S-DVB)-*g*-P(S-GMA)-TEDETA beads (0.1 g) were introduced to the assay mixture to initiate the reaction as above. After 10 min, the reaction was terminated by removal of the beads from the reaction mixture. The absorbance of the reaction mixture was determined and the immobilized catalase activity was calculated. These activity assays were carried out over the pH range of 4.0–9.0 and temperature range of 15–55 °C to determine the pH and temperature profiles for the free and immobilized enzyme. The effect of substrate concentration was tested in the 5–30 mM H_2O_2 concentrations range. The results of pH and temperature are presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.8. Operational, thermal and storage stabilities of catalase preparations

The activity of free and immobilized catalase after storage in phosphate buffer (50 mM, pH 7.0 at 4 °C was measured for eight weeks in a batch-mode as given above.

The operational stability of immobilized enzyme in terms of repetitive uses was performed in 25 successive measurements in the same day.

The tests for the determination of the thermal stability of free and immobilized catalase were carried out by measuring the residual activity of the enzyme, exposed to two different temperatures (55 and 65 °C) in phosphate buffer (50 mM, pH 7.0) for 120 min. A predetermined time interval, a sample was removed and assayed for enzymatic activity as described above.

2.9. Reusability of the catalase immobilized P(S-DVB)-g-P(S-GMA)-TEDETA beads

In order to determine the reusability of the catalase immobilized P(S-DVB)-*g*-P(S-GMA)-TEDETA beads, enzyme adsorption and desorption cycle was repeated five times. Desorption experiments were carried out in acetate buffer solution containing

1.0 M NaSCN at pH 4.0. Catalase-immobilized P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was placed in the desorption medium for 2 h with a stirring rate at 150 rpm, and at 25 °C. The beads were washed several times with phosphate buffer (50 mM, pH 7.0), and were then reused in enzyme immobilization. Catalase concentration within the desorption medium was determined as described above.

2.10. Characterization of beads

The bromide content of the beads was determined by simple titration. For this purpose, 0.2 g of the bromoacetylated polymer was mixed with 10 mL 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 calorimetrically by mercuric thiocyanate method (Furniss et al., 1978). This analysis gave 0.84 mmol bromine per gram of the polymer. Determination of the amine content of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was carried out by potensiometric titration. Briefly, the beads sample (1.0 g) was mixed with HCl acid (15 mL, 1.0 M) in a flask and stirred for overnight. The mixture was filtered and 2 mL of the filtrate was titrated with NaOH (0.1 M) in the presence of phenolphthalein.

The specific surface area of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was measured by a surface area apparatus and calculated using the BET method (Brunauer et al., 1938). The density of P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was determined by using a stereopycnometer (Quantachrome, SPY 3, USA).

The Fourier transform infrared (FTIR) spectra were carried 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) and modified counterpart beads (0.01 g) and KBr (0.1 g) were thoroughly mixed and the mixture was pressed to form a tablet, and the spectrum was recorded.

The surface morphology of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was observed by scanning electron microscopy (SEM). The dried beads were coated with gold under reduced pressure and their electron micrographs were obtained using a scanning electron microscope (JEOL, JSM 5600, Japan).

3. Results and discussion

3.1. Properties of the multi-modal ligand incorporated beads

The crucial task in SI-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 appropriate functional co-monomer. Non-hydrolysibility 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 common approach and has been widely employed by many authors (Carrot et al., 2001). However, hydrolysis of this bond chemically or enzymatic means deters the use of this approach when the hairy carrier polymer is considered to recycle and reuse. The surface halogen atoms (i.e., Br) of the P(S-DVB) beads were successfully used as the initiating groups for surface initiated atom transfer radical polymerization. SI-ATRP-based initiators (i.e., H-TETA and CuBr) were used for SI-ATRP of two different polymers (Supplementary Fig. S1) (Karagoz et al., 2010). Firstly, PS was grafted to the P(S-DVB) beads and then P(GMA) was then introduced by followed SI-ATRP from terminal bromoalkyl groups of the PS grafts. The chemical transformations in each step were followed by monitoring characteristic bands in FT-IR spectra of the products (Supplementary Fig. S2). In the spectrum of P(S-DVB-Br) (the product of Step 2; Supplementary Fig. S2a) bearing bromoacetyl groups, typical stretching vibration band of the carbonyl (C=O) group is observed at 1719 cm^{-1} as a weak band. Apparently, low intensity of this band is due to 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 3; Supplementary Fig. S2b) the later 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} becomes also 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 4; Supplementary Fig. S2c). The typical bands arising from the epoxy functionality are not discernible around 1000 cm^{-1}, because this region is over crowded contains many bands. Nevertheless, the shoulder around 1170 cm^{-1} can be ascribed to 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 at 3100–3500 cm^{-1} range can be considered as O–H vibration band arising from ring opening of the epoxy function (the product of Step 4, Supplementary Fig. S2c). Incorporation of TEDETA on P(GMA) in the last step (Step 5, Supplementary Fig. S2d), does not induce marked change in the FT-IR spectrum of P(SDVB)-*g*-P(S-d-GMA)-TEDETA. This must be due to the fact that, this modification does not bring new group exhibiting sharp bands. Nevertheless, the weak band around 970 cm^{-1} can be assigned to C–N bond, which is formed by reaction of NH group of TEDETA with the epoxy unit. Another change is emerging of a very broad band in 3100–3500 cm^{-1} range can be considered as O–H vibration band arising from ring opening of the epoxy function.

Scanning electron microscopy (SEM) micrographs presented in Supplementary Fig. S3 shows a smooth surface structure of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads. The non-porous surface properties of the beads would reduce diffusion limitation of the substrate and product during enzymatic decomposition reactions of hydrogen peroxide.

The specific surface area of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads was measured by BET method and was found to be 14.6 m^2/g beads. The amount of epoxy groups on the P(S-DVB)-*g*-P(S-GMA) beads was determined to be 0.18 mmol/g beads. The multi modal-ligand content of the P(S-DVB)-*g*-P(S-GMA)-TEDETA beads were determined via potensiometric titration. NaOH consumption was determined as 18.6 mL. The titration results revealed that P(S-DVB)-*g*-P(S-GMA)-TEDETA beads has 1.05 mmol amine group or else 0.35 mmol ligand units per gram of the polymer.

3.2. Immobilization of catalase on the P(S-DVB)-g-P(S-GMA)-TEDETA beads

3.2.1. Effect of pH on catalase adsorption capacity

The pH value of the solution affects both external charge distribution of catalase molecules and the functional secondary amine groups of the ligand incorporated beads. In order to investigate the effects of pH on the catalase adsorption efficiency and capacity of P(S-DVB)-*g*-P(S-GMA)-TEDETA beads, the adsorption behaviors of catalase on TEDETA functionalized beads were investigated at pH between pH 3.0 and 8.0 in appropriate buffer. As shown in Supplementary Fig. 4. The maximum binding capacity was observed at around pH 6.5 and gave the highest adsorption capacity. TEDETA is

a multi-modal ligand, the optimal adsorption with multi-modal ligand was generally obtained at around the p*I* value of the target protein. Since the p*I* value of catalase is 6.5. The maximum catalase adsorption was obtained at pH 6.5, and similar observations are reported in the literature for various multi-modal ligands (Yavuz et al., 2009; Yang et al., 2007). For a multi-modal ligand, a mixed type interaction is observed between ligand and target protein such as electrostatic and hydrophobic binding interactions (Fig. 1). The TEDETA multi-modal ligand possessing three tertiary amine and four hydrophobic ethyl groups that can interact with the target molecule (i.e., catalase) in different ways, thus, a mixed mode interaction can be obtained. In addition, the polystyrene segment of the fibrous polymer has several aromatic rings for hydrophobic interaction with the target enzyme molecules. Thus, these interactions between catalase and TEDETA-functionalized beads at pH 6.5 may result from both the conformational state of the functional groups on the grafted polymer chains and amino acid side chains of the catalase molecule. In addition, during the epoxy ring opening reaction a hydroxyl group was formed in the proximity of the TEDETA ligand. Thus, the relative position of the hydroxyl groups on the ligand incorporated polymer chains could also provide an additional hydrogen bonding sites for the target biomolecules. Thus, a created specific binding side could also improve the adsorption capacity of catalase. Proteins that change conformation as a function of their environment (pH, salt, temperature, etc.), such as catalase, it has a molecular mass of 250 kDa, and it could change conformational structure upon binding on a functional surface. Thus, catalase molecules would expand and contact according to the variation of the ionizable groups on its surfaces. As medium pH rises, the TEDETA ligand incorporated polymer chains are closely packed, limiting the interaction of catalase with functional groups, thus, a decrease in adsorption capacity will be observed for TEDETA-incorporated beads.

3.2.2. Effect of ionic strength on adsorption capacity

The enthalpy of adsorption would be affected not only by the pH value on the electron donating capability, but also by the salt concentration on the hydrophobic and electrostatic interaction between catalase and the functional groups of beads. As seen in Supplementary Fig. S5, the adsorption capacity of the TEDETA ligand incorporated beads was significantly changed for catalase with increasing NaCl concentration from 0 to 1.5 M as the ionic strength increases. A similar observation was reported previously (Johansson et al., 2003), they studied the interaction of several multi-modal ligands with negatively charged biomolecules. Different type multimodal ligands were immobilized on Sepharose 6 support and the adsorption properties of the negatively charged protein BSA was studied under different NaCl concentrations. They reported that the non-aromatic multi-modal ligands based on amines groups are optimal for the capture of proteins at high salt conditions. They also suggested that these new multi-modal anion-exchange ligands could be designed to take advantage not only

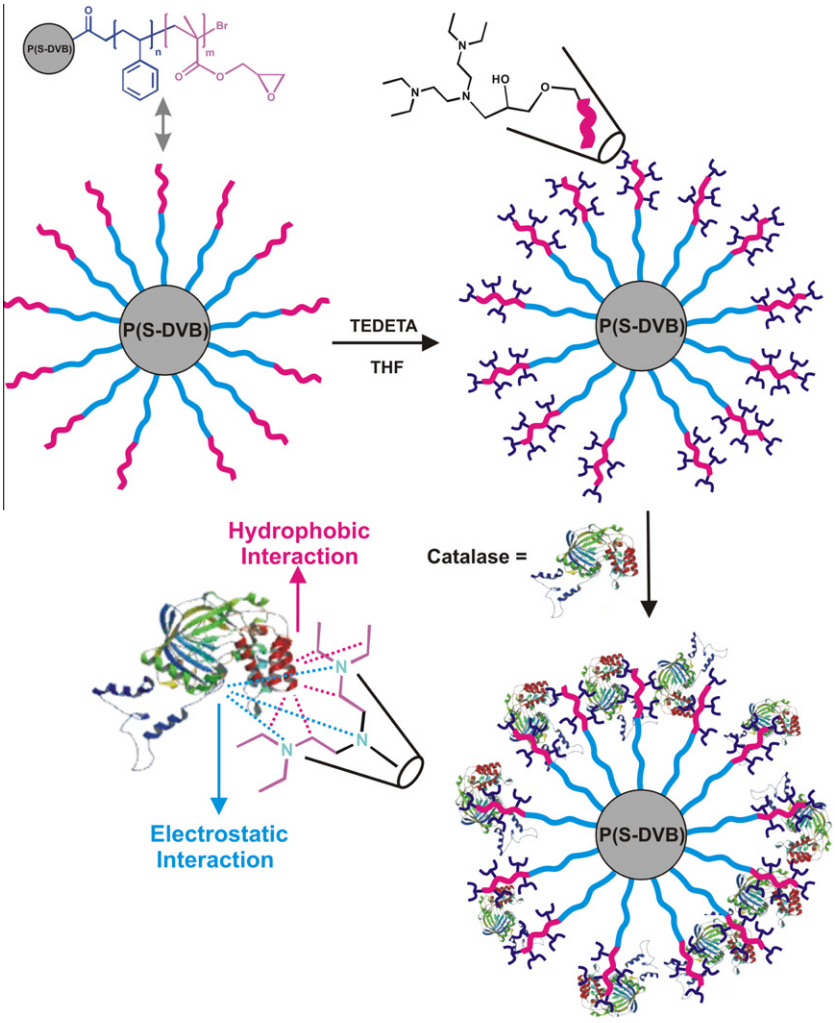

Fig. 1. Chemistries used for the immobilization of catalase via adsorption on the P(S-DVB)-*g*-P(S-b-GMA)-TEDETA ligand.

of electrostatic but also of hydrogen bond interactions. In addition, the biomolecules to be separated are negatively charged which means that they can exhibit hydrogen acceptor properties. Further, the adsorption of catalase remained high even under high salt condition. This may indicate that non-electrostatic interactions (e.g., hydrophobic, hydrogen bonding) may become dominant for catalase on the TEDETA ligand attached beads at higher salt concentrations. Thus, any functional group in the ligand, able to participate in hydrogen bond interaction as an donor, should be able to generate the extra mode of interaction required to obtain high breakthrough capacities at high ionic-strength conditions (Johansson et al., 2003; Yavuz et al., 2009).

3.2.3. Effect of initial concentration of catalase

Fig. 2 shows the adsorption capacity of P(S-DVB)-*g*-P(S-GMA)-TEDETA beads by investigating the efficiency of immobilization at different enzyme concentrations in phosphate buffer (50 mM, pH 6.5) at 15 °C. From the equilibrium adsorption observations, it can be concluded that the amount of catalase adsorbed by the TEDETA ligand attached beads increased with increasing catalase concentration in the medium. As presented in Fig. 2 with increasing enzyme concentration in solutions, the amount of catalase adsorbed per unit area by TEDETA ligand attached beads increases almost linearly all the tested enzyme concentration range (up to 3.0 mg/mL). The maximum catalase immobilization onto TEDETA ligand attached beads was found to be 84.1 mg/g with 3.0 mg/mL catalase initial concentration. The specific interactions between catalase and the TEDETA ligand attached beads can result from the co-operative effect of different mechanisms such as specific interaction (i.e., hydrogen bonding, hydrophobic interaction) caused by the hydroxyl, carboxyl groups and hydrophobic group of the catalase amino acid residues with the multi-modal ligand of the beads.

3.3. Adsorption isotherms

Three theoretical isotherm models were used to fit the experimental data: Langmuir, Freundlich and Temkin model (Temkin and Pyzhev, 1940). The Langmuir model is based on assumption homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. Since the Langmuir model is formulated for homogenous adsorption. The Freundlich and Temkin isotherm models are usually adopted for heterogeneous adsorption. The Freundlich isotherm is frequently used to describe the adsorption. It relates the adsorbed concentration as the power function of solute concentration. One limitation of the Freundlich model is that the amount of adsorbed solute increases indefinitely with the concentration of solute in the solution. This empirical equation takes the form:

$$Q_{eq} = K_F (C_{eq})^{1/n} \quad (2)$$

where K_F and n are the Freundlich constants characteristic of the system. K_F and n are indicator of the adsorption capacity and adsorption intensity, respectively. The slope and the intercept of the linear Freundlich equation are equal to $1/n$ and $\ln K_F$, respectively.

The Temkin isotherm model describes the behavior of many adsorption systems on heterogeneous surface and it is based on the following equation:

$$q = q_T \ln(1 + K_T C) \quad (3)$$

where K_T (mL/mg) is the equilibrium binding constant corresponding to the maximum binding energy ($K_T = \exp(-\Delta G_{max}/RT)$), C (mg/mL) is the concentration of protein in the solution at equilibrium, q (mg protein/g beads) is the amount of protein adsorbed on the beads surface, and q_T (mg protein/g beads) is the differential surface capacity for protein adsorption per unit binding energy. In the case of Temkin-type fit the experimental data, the semilogarithmic plot of $\ln C_{eq}$ versus q_{eq} was employed to generate the intercept value of $\ln K_T$ and the slope of q_T.

The corresponding semi-reciprocal plot and Scatchard plot gave a non-linear plot for the TEDETA ligand attached beads. In other words, a non-linear Scatchard plot indicates the adsorption heterogeneity (Arica et al., 2004). Since the adsorption of catalase onto TEDETA ligand attached beads cannot be described in terms of the Langmuir model. The magnitude of K_F and n values of Freundlich model showed easy uptake of catalase from aqueous medium with a high adsorption capacity of the TEDETA ligand attached beads. Values of $n > 1$ for TEDETA ligand attached beads indicate positive cooperativity in binding and a heterogeneous nature of adsorption (Table 1).

In the case of the Temkin model, the corresponding semi-logarithmic plot gave rise to linear plot for the binding of catalase to TEDETA ligand attached beads and the correlation coefficient of the semi-logarithmic plots (R^2) was above 0.990 for the TEDETA ligand attached beads, indicating the Temkin model fitted the experimental data. The fitted parameter values for Temkin model are presented in Table 1. For the adsorption process of a protein, the possible binding sites can be non-specific or specific. All these interactions between catalase and TEDETA ligand attached beads should result in uniform binding energies, up to some maximum binding energy (ΔG_{max}). In these cases, ΔG_{max} values were found to be −2.64 and −4.27 kJ/mol for bare P(S-DVB)-*g*-P(S-b-GMA) beads and ligand attached P(S-DVB)-*g*-P(S-b-GMA)-TEDETA beads, respectively. The ΔG_{max} value of TEDETA ligand attached beads for catalase adsorption was smaller than that of the bare P(S-DVB)-*g*-P(S-b-GMA) beads (Table 1). These results indicate that there is a relationship between the surface interactions groups of catalase with the presented bare and TEDETA ligand attached beads. From these observations, the range and distribution of binding energies should depend on the density, and type of functional groups, both on the protein and the adsorbent surface. As indicated in the Temkin model, the binding energy decreased with increasing the amounts of adsorbed protein on the adsorbents surface (Table 1).

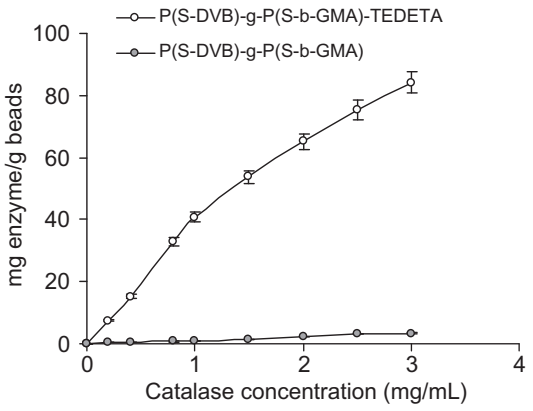

Fig. 2. Effect initial catalase concentration on the immobilization efficiency of the catalase on the TEDETA attached beads.

3.4. Effect of experimental variables

The effect of pH on the activity of the free and immobilized catalase for degradation of hydrogen peroxide was examined in the pH range 4.0–9.0 at 35 °C. As seen from Fig. 3, the degradation reaction has maximum activity free and immobilized enzymes at

Table 1
The Temkin and the Freundlich models parameters for adsorption isotherms.

	Freundlich			Temkin			
	n	K_F	R^2	K_T (mg/mL)	q_T (mg/g)	R^2	ΔG (kJ/mol)
P(S-DVB)-g-P(S-b-GMA)	0.71	0.92	0.997	2.92	1.46	0.966	−2.64
P(S-DVB)-g-P(S-b-GMA)-TEDETA	1.14	42.01	0.991	5.59	28.40	0.990	−4.27

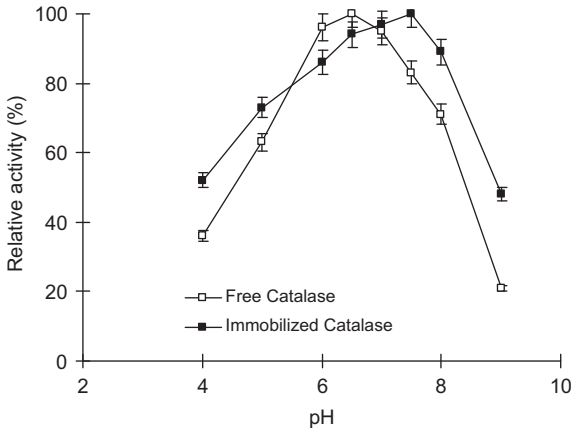

Fig. 3. Effect of pH on the activity of free and immobilized catalase on the TEDETA attached beads; the relative activities at the optimum pH were taken as 100% for free and immobilized catalase, respectively.

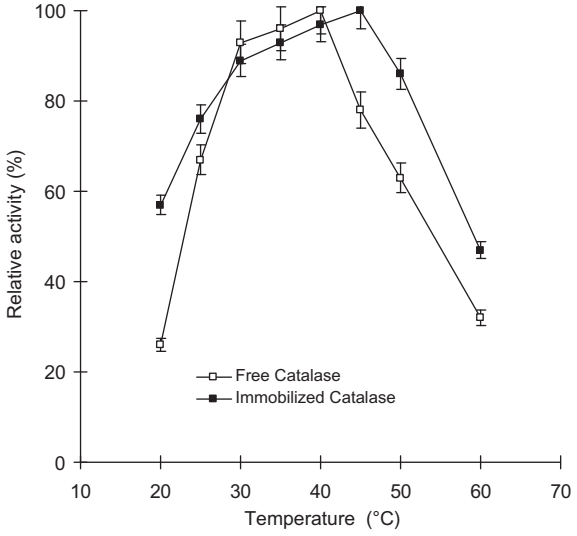

Fig. 4. Effect of temperature on the activity of the free and immobilized catalase on the P(S-DVB)-g-P(S-b-GMA)-TEDETA beads; the relative activities at the optimum temperature were taken as 100% for free and immobilized catalase, respectively.

pH 6.5 and at pH 7.5, respectively. This shift may depend on the immobilization method as well as the basic character of the support material. The pH profiles of the immobilized catalase display strongly improved stability of the optimum pH value, in comparison to that of the free form, which means that the immobilization method preserved the enzyme activity (Arica, 2000).

Fig. 4 shows the effect of temperature in the initial rate of hydrogen peroxide decomposition for free and immobilized catalase. Free catalase shows the maximum activity at 40 °C, whereas, the immobilized catalase reaches at 45 °C. As was evident from the data, the immobilized enzyme possessed a better heat-resistance than that of the free enzyme. The immobilization of catalase on the fibrous polymer via electrostatic and hydrophobic interaction might also reduce the conformational flexibility and may result in higher activation energy for the molecule to reorganize the proper conformation for the binding to substrate hydrogen peroxide.

3.5. Kinetic parameters for free and immobilized catalase

Kinetic parameters K_m and V_{max} for the hydrogen peroxide degradation were calculated from Lineweaver–Burk plots at constant temperature and pH while varying the substrate concentration. K_m values were calculated as 0.25 ± 0.03 and 0.43 ± 0.02 mM for the free and immobilized enzyme, respectively. The change in the affinity of the enzyme to its substrate is probably caused by the multipoint interactions of the enzyme introduced by the immobilization via adsorption. The K_m value of the immobilized catalase was increased compared to free counterpart, possibly due to the diffusional limitation imposed on the flow of substrate molecules from of the fibrous layers of the grafted polymer. This may be explained by the fact that fibrous polymer chains of the beads should be covered with adsorbed catalase, resulting a sticky web-like coating over the fibrous polymer surface. Thus, these layers may prevent the diffusion of the substrate molecules from the inner layer of the polymer brushes where the enzyme was immobilized (Arica et al., 2004). The V_{max} values of the free and immobilized enzyme were estimated from the experimental data as 3925 ± 23 and 3560 ± 37 U/mg proteins, respectively. As expected, the K_m and V_{max} values were significantly affected after immobilization of catalase on to the TEDETA attached beads.

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

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

where $v_{\text{immobilized}}$ and v_{free} were the reaction rate of the immobilized and free enzyme, respectively. From this calculation, TEDETA ligand attached system provided an efficiency factor of 0.907 ± 0.033 for the immobilized catalase. 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 catalase were found to be 15496 and 8093, respectively. The catalytic efficiency of catalase was decreased about 1.9-fold upon immobilization. The retained catalase activity was obtained up to 86.2% in this study, and is comparable with the related literature (Jurgen-Lohmann and Legge, 2006; Bayramoglu and Arica, 2010).

3.6. Stability studies

An important advantage of enzyme immobilization is the stability improvement, which expands the range of conditions suitable for enzyme function in industrial applications. The storage stability of immobilized catalase in phosphate buffer (50 mM, pH 7.0) is much better than that of the free catalase at 4 °C. The half-life of free catalase is three week at 4 °C, while that of the immobilized catalase half-life is around seven weeks (data not shown). A similar observation was reported for immobilized catalase on the different supports (Bayramoglu and Arica, 2010; Alptekin et al., 2010).

The successful application of the immobilized enzymes in the industrial area requires not only high storage stabilities but also

high operational stabilities. The operational stability of the immobilized catalase was determined at optimum activity conditions by using enzyme immobilized on the TEDETA ligand attached beads in 25 activity assays in 1 day. An activity loss of 12% was observed within the first five uses (in this case, a small amount of enzyme leakage was detected in the reaction medium and wash solutions. However, after these five uses no enzyme leakage was detected from the support) and remained constant for another 15 measurements. After 25 cycle repeated uses, the immobilized enzyme retained about 78% of its initial activity. The activity loss may be due to enzyme deactivation during repeated uses (data not shown).

The effect of temperature on the stability of the free and immobilized catalase was shown in Fig. 5. The pattern of heat stability indicated that a smaller rate of thermal inactivation was observed for the immobilized catalase on the TEDETA ligand attached beads than that of the free enzyme. At 55 °C, the free enzyme lost about 73% its initial activity after 120 min of heat treatment, while the immobilized enzyme showed significant resistance to thermal inactivation (lost about 6% of its initial activity in the same period). At 65 °C, the free catalase lost all its initial activity after 60 min heat treatment. Under the same conditions, the immobilized catalase retained about 36% of its initial activity. These results suggest that the thermostability of immobilized catalase becomes significantly higher at higher temperature. If the heat stability of enzymes increased upon immobilization, the potential application of these enzymes would be extended. In a previous study, bovine liver catalase was covalently immobilized onto Eupergit C. The thermal stability of the immobilized catalase was comparably higher than that of the free catalase at 40 °C (Alptekin et al., 2010).

3.7. Regeneration of the support for reuse in enzyme immobilization

The cleaning of adsorbed catalase after deactivation from the TEDETA ligand attached beads was achieved under acidic condition. The catalase adsorbed on the TEDETA ligand attached beads was placed within the cleaning medium containing 1.0 M NaSCN in acetate buffer pH 4.0. The adsorption–desorption cycle of catalase was repeated five times by using the beads (Fig. 6). The immobilization capacity of the affinity beads did not change significantly after five times use in the repeated use of the support after regeneration of the beads in cleaning medium for 2 h. The fifth adsorption–desorption cycle of catalase, the amount of immobilized enzyme (39.3 ± 1.3 mg/g beads) was about 4% lower than that of the first use (40.8 ± 1.7 mg protein/g beads). This indicates that the prepared TEDETA attached beads were of high stability in repeated enzyme loading. Thus, the TEDETA attached beads can be reused in enzyme immobilization process with significant cost saving. In a previous study, catalase was immobilized on the poly(itaconic acid) grafted chitosan membrane, and at the end of the sixth cycles, catalase immobilization capacity of the membrane was decreased about 7%. The results are comparable with the earlier reported study (Bayramoglu and Arica, 2010).

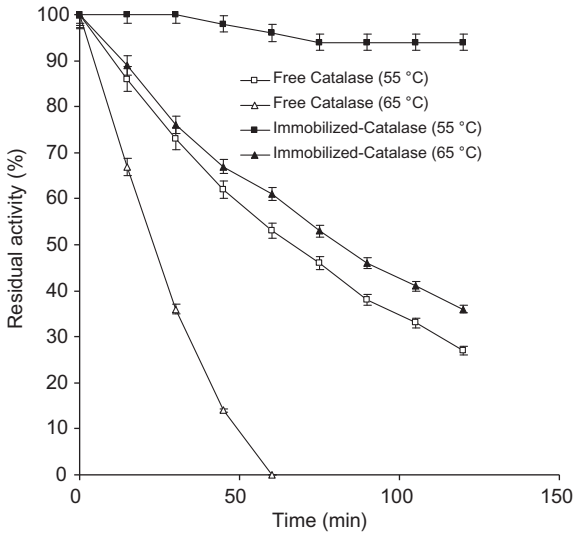

Fig. 5. Thermal stability of the free and immobilized catalase at two different temperatures.

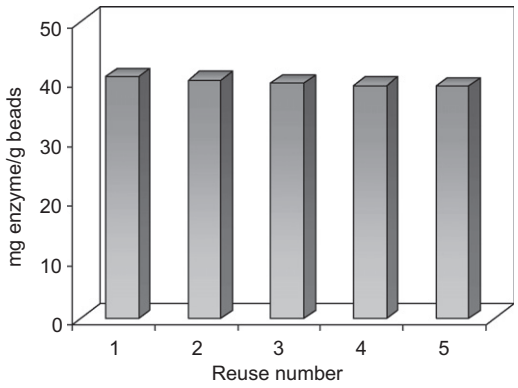

Fig. 6. Reuseability of the TEDETA attached beads in catalase immobilization.

4. Conclusions

Hydrophobic/hydrophilic di-block polymers grafted P(S-DVB) and TEDETA ligand attached beads was used for catalase immobilization. The experimental data showed that the desired amount of enzyme could be loaded on the beads by changing the initial concentration of enzyme in the medium. The fibrous polymer grafted and ligand attached supports can provide a high specific surface area with a large catalase immobilization capacity, the quantity and the accessibility of the active sites on the fibrous polymer necessary for high reaction rates and conversions. Finally, P(S-DVB-*g*-P(S-GMA)-TEDETA beads prepared in this work showed promising potential for various biotechnological applications.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.12.029.

References

Acar, M.H., Bicak, N., 2003. Synthesis of hexylated triethylenetetramine. A new ligand for homogeneous atom transfer radical polymerization. J. Polym. Sci. Part A: Polym. Chem. 41, 1677–1680.

Alptekin, O., Tukel, S.S., Yıldırım, D., Alagöz, D., 2010. Immobilization of catalase onto Eupergit C and its characterization. J. Mol. Catal. B: Enzym. 64, 177–183.

Arica, M.Y., Bayramoglu, G., 2004. Reversible immobilization of tyrosinase onto polyethyleneimine-grafted and Cu(II) chelated poly(HEMA-co-GMA) reactive membranes. J. Mol. Catal. B: Enzym. 27, 255–265.

Arica, M.Y., Yilmaz, M., Yalin, E., Bayramoglu, G., 2004. Affinity membrane chromatography: relationship of dye-ligand type to surface polarity and their effect on lysozyme separation and purification. J. Chromatogr. B 805, 315–323.

Arica, M.Y., Bayramoglu, G., 2006. Invertase reversibly immobilized onto polyethylenimine-grafted poly(GMA-MMA) beads for sucrose hydrolysis. J. Mol. Catal. B: Enzym. 38, 131–138.

Arica, M.Y., Soydogan, H., Bayramoglu, G., 2010. Reversible immobilization of *Candida rugosa* lipase on fibrous polymer grafted and sulfonated p(HEMA/EGDMA) beads. Bioprocess. Biosyst. Eng. 33, 227–236.

Arica, M.Y., 2000. Immobilization of polyphenol oxidase on carboxymethylcellulose hydrogel beads: preparation and characterization. Polym. Int. 49, 775–781.

Arica, M.Y., Altintas, B., Bayramoglu, G., 2009. Immobilization of laccase onto spacer-arm attached non-porous poly(GMA/EGDMA) beads: application for textile dye degradation. Bioresour. Technol. 100, 665–669.

Arica, M.Y., Tunalı, Y., Bayramoglu, G., 2007. Immobilization of β-galactosidase onto magnetic poly(GMA-MMA) beads for hydrolysis of lactose in bed reactor. Catal. Commun. 8, 1094–1101.

Bayramoglu, G., Altintas, B., Yilmaz, M., Arica, M.Y., 2010. Immobilization of chloroperoxidase onto highly hydrophilic polyethylene chains via bio-conjugation: catalytic properties and stabilities. Bioresour. Technol. 102, 475–482.

Bayramoglu, G., Arica, M.Y., 2010. Reversible immobilization of catalase on fibrous polymer grafted and metal chelated chitosan membrane. J. Mol. Catal. B: Enzym. 62, 297–304.

Bayramoglu, G., Yilmaz, M., Arica, M.Y., 2010a. Reversible immobilization of laccase to poly(4-vinylpyridine) grafted and Cu(II) chelated magnetic beads: biodegradation of reactive dyes. Bioresour. Technol. 101, 6615–6621.

Bayramoglu, G., Arica, M.Y., 2009. Immobilization of laccase onto poly(glycidylmethacrylate) brush grafted poly(hydroxyethylmethacrylate) films: enzymatic oxidation of phenolic compounds. Mater. Sci. Eng. C 29, 1990–1997.

Bayramoglu, G., Senel, A.U., Yilmaz, M., Arica, M.Y., 2008. Preparation of nanofibrous polymer grafted magnetic poly(GMA-MMA)-*g*-MAA beads for immobilization of trypsin via adsorption. Biochem. Eng. J. 40, 262–274.

Bhattacharya, A., Misra, B.N., 2004. Grafting a versatile means to modify polymers-techniques, factors and applications. Prog. Polym. Sci. 29, 767–814.

Bicak, N., Gazi, M., Galli, G., Chiellini, E., 2006. Polystyrene microspheres having epoxy functional dangling chains linked by hydrolytically stable bonds via ATRP. J. Polym. Sci. Part A: Polym. Chem. 44, 6708–6716.

Boland, M.J., Hesselink, P.G.M., Hustedt, H., 1989. Extractive purification of enzymes from animal tissue using aqueous phase systems. J. Biotechnol. 11, 337–352.

Bolivar, J.M., Mateo, C., Rocha-Martin, J., Cava, F., Berenguer, J., Fernandez-Lafuente, R., Guisan, J.M., 2009. The adsorption of multimeric enzymes on very lowly activated supports involves more enzyme subunits: stabilization of a glutamate dehydrogenase from *Thermus thermophilus* by immobilization on heterofunctional supports. Enzyme Microb. Technol. 44, 139–144.

Brunauer, S., Emmett, P.H., Teller, E., 1938. Adsorption of gases in multimolecular layers. J. Am. Chem. Soc. 60, 309–319.

Carrot, G., Diamanti, S., Manuszak, M., Charleux, B., Vairon, J.-P., 2001. Atom transfer radical polymeriztion of *n*-butyl acrylate from silica nanoparticles. J. Polym. Sci. Part A: Polym. Chem. 39, 4294–4301.

Chang, Y.-K., Chou, S.-Y., Liu, J.-L., Tasi, J.-C., 2007. Characterization of BSA adsorption on mixed mode adsorbent I. Equilibrium study in a well-agitated contactor. Biochem. Eng. J. 35, 56–65.

Cheng, Z.F., Wu, C.M., Yang, W.H., Xu, T.W., 2010. Preparation of bromomethylated poly(2,6-dimethyl-1,4-phenylene oxide) hollow fiber cation-exchange membranes and immobilization of cellulase thereon. J. Membr. Sci. 358, 93–100.

Cullen, S.P., Liu, X.S., Mandel, I.C., Himpsel, F.J., Gopalan, P., 2008. Polymeric brushes as functional templates for immobilizing ribonuclease A: study of binding kinetics and activity. Langmuir 24, 913–920.

Furniss, B.H., Hannaford, A.J., Rogers, V., Smith, P.W.G., Tatchell, A.R. (Eds.), Vogel's Textbook of Practical Organic Chemistry, fourth ed. Longman Inc., New York, 1978, p. 287.

Jain, P., Vyas, M.K., Geiger, J.H., Baker, G.L., Bruening, M.L., 2010. Protein purification with polymeric affinity membranes containing functionalized poly(acid) brushes. Biomacromolecules 11, 1019–1026.

Johansson, B.L., Belew, M., Eriksson, S., Glad, G., Lind, O., Maloisel, J.L., Norrman, N., 2003. Preparation and characterization of prototypes for multi-modal separation media aimed for capture of negatively charged biomolecules at high salt conditions. J. Chromatogr. A 1016, 21–33.

Jurgen-Lohmann, D.L., Legge, R.L., 2006. Immobilization of bovine catalase in sol–gels. Enzyme Microb. Technol. 39, 626–633.

Karagoz, B., Bayramoglu, G., Altintas, B., Bicak, N., Arica, M.Y., 2010. Poly(glycidyl methacrylate)-polystyrene diblocks copolymer grafted nanocomposite microspheres from surface-initiated atom transfer radical polymerization for lipase immobilization: application in flavor ester synthesis. Ind. Eng. Chem. Res. 49, 9655–9665.

Lei, Z., Ren, N., Li, Y., Li, N., Mu, B., 2009. $Fe3O_4/SiO_2$-g-PSStNa polymer nanocomposite microspheres (PNCMs) from a surface-initiated atom transfer radical polymerization (SI-ATRP) approach for pectinase immobilization. J. Agric. Food Chem. 57, 1544–1549.

Sun, L., Dai, J.H., Baker, G.L., Bruening, M.L., 2006. High-capacity, protein-binding membranes based on polymer brushes grown in porous substrates. Chem. Mater. 18, 4033–4039.

Temkin, M.I., Pyzhev, V., 1940. Kinetics of ammonia synthesis on promoted iron catalysts. Acta Physiochim. USSR 12, 217–222.

Torres, R., Pessela, B., Fuentes, M., Munilla, R., Mateo, C., Fernandez-Lafuente, R., Guisan, J.M., 2005. Stabilization of enzymes by multipoint attachment via reversible immobilization on phenylboronic activated supports. J. Biotechnol. 120, 396–401.

Wang, L., Wei, L., Chen, Y., Jiang, R., 2010. Specific and reversible immobilization of NADH oxidase on functionalized carbon nanotubes. J. Biotechnol. 150, 57–63.

Xu, F.J., Neoh, K.G., Kang, E.T., 2009. Bioactive surfaces and biomaterials via atom transfer radical polymerization. Progr. Polym. Sci. 34, 719–761.

Yang, T., Malmquist, G., Johansson, B.-L., Maloisel, J.-L., Cramer, S., 2007. Evaluation of multi-modal high salt binding ion exchange materials. J. Chromatogr. A 1157, 171–177.

Yavuz, E., Bayramoglu, G., Senkal, B.F., Arica, M.Y., 2009. Poly(glycidylmethacrylate) brushes generated on poly(VBC) beads by SI-ATRP technique: hydrazine and amino groups functionalized for invertase adsorption and purification. J. Chromatogr. B 877, 1479–1486.

Zhou, J., 2010. Immobilization of cellulase on a reversibly soluble–insoluble support: properties and application. J. Agric. Food Chem. 58, 6741–6746.