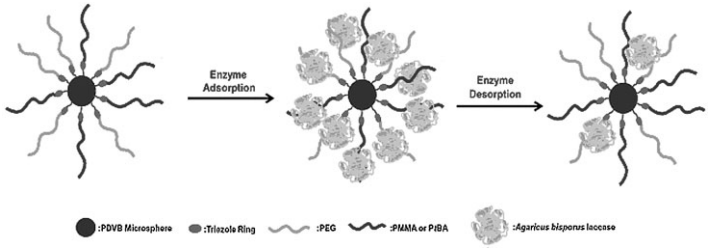
Modification of Polydivinylbenzene Microspheres by a Hydrobromination/Click-Chemistry Protocol and their Protein-Adsorption Properties

Yasemin Yuksel Durmaz, Bunyamin Karagoz, Niyazi Bicak, Dilek Odaci Demirkol, Esra Evrim Yalcinkaya, Suna Timur,* Yusuf Yagci*

Hydrophobic- and/or hydrophilic-polymer-grafted PDVB microspheres are synthesized by the combination of hydrobromination and click-chemistry processes. The modified-PDVB microspheres and the intermediates at various stages of synthesis are characterized using GPC, ^{1}H NMR and FTIR spectroscopy and TGA analysis. Use of the microspheres as a support matrix for reversible protein immobilization via adsorption is investigated. The system parameters such as the adsorption conditions (i.e., enzyme concentration, medium pH) and desorption are studied and evaluated with regards to the biocatalytic activity and adsorption capacity.



Introduction

The screening of a suitable matrix is very important in protein adsorption and separation. Polymer colloids, largely used in coatings, adhesive, or inks, have recently found interest in medical and biological applications such as bioseparation, immunoassay, and affinity diagnosis, or as a carrier for drug-delivery purposes. All of these applications require suitable particle sizes, nature and density of the surface functional groups, and colloidal stability.[1]

Enzyme adsorption has been a popular strategy for most large-scale applications due to the ease in catalyst recycling, continuous operation, and product purification. However poor biocatalytic efficiency of the adsorbed enzymes often limits the development of large-scale bioprocessing to compete with traditional chemical processes. Improvements in the biocatalytic efficiency can be achieved by manipulating the structure of the carrier materials for biomolecule adsorption. The most-important advantages of this method are the stability of the enzyme activity after adsorption and the reuse of the enzyme and support material for different purposes, because of the reversibility of the method.[2] As support matrices, polymeric microspheres have attracted much attention because they can be produced easily in a wide variety of compositions and can be modified for the immobilization of systems by introducing a variety of activation methods.[3]

Polymerization methods, including water-based emulsion, seeded suspension, non-aqueous dispersion polymer-

Y. Y. Durmaz, B. Karagoz, N. Bicak, Y. Yagci
Istanbul Technical University, Faculty of Science and Letters, Department of Chemistry, Maslak, 34469, Istanbul, Turkey
Fax: +90 212 285 6386; E-mail: yusuf@itu.edu.tr
D. O. Demirkol, S. Timur
Ege University, Faculty of Science, Biochemistry Department, Bornova, 35100, Izmir, Turkey
Fax: +90 232 343 8624; E-mail: suna.timur@ege.edu.tr
E. E. Yalcinkaya
Ege University, Faculty of Science, Chemistry Department, Bornova, 35100, Izmir, Turkey

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ization, and precipitation polymerization, have been successfully employed for the preparation of monodisperse microspheres.[4–7] Among them, precipitation polymerization, which can be performed in the absence of any added surfactant or stabilizer,[8–11] appears to be an attractive route to obtain microspheres with uniform size and shape. Typically, monodisperse and highly crosslinked polydivinylbenzene (PDVB) surfactant-free microspheres (with diameters between 2–5 μm) have been prepared by using only divinylbenzene (DVB), 2,2'-azoisobutyronitrile (AIBN), and acetonitrile as a monomer, radical initiator and solvent, respectively.[8] Interestingly, PDVB microspheres formed in this method contain a significant amount of residual double bonds on the surface of the particle.[12] The residual double bonds located at the surface permit further growth and modification of the particles. The properties of the polymeric particles are considerably changed by surface modification, which is frequently achieved in a core/shell fashion through the incorporation of functional groups and new monomer(s). Within the past decade, the development of controlled/living radical polymerization (CLRP) methods,[13–17] and new synthetic coupling routes have allowed the functionalization of polymer particles. As a result, a great variety of monomers grafted on PDVB microspheres using CLRP methods, including reversible addition/fragmentation chain transfer (RAFT)[18] and atom-transfer radical polymerization (ATRP)[19–21] processes are now available. Very recently, a combination of reversible-addition fragmentation chain-transfer polymerization and hetero Diels-Alder (HDA) chemistry was utilized to successfully generate functional core/shell microspheres by Barner and co-workers.[22] In addition, facile access to hydroxyl-functional core/shell microspheres via grafting of ethylene oxide by anionic ring-opening polymerization was recently reported by Joso et al.[23]

"Click reactions",[24–26] in particular Cu(I)-catalyzed 1,3-dipolar Huisgen cycloaddition reactions between an azide and an alkyne, have gained a great deal of attention due to their high specificity and nearly quantitative yields in the presence of many functional groups. This coupling process has been widely used for the modification of polymeric materials.[27–38] Recently, PDVB microspheres were functionalized[39] by polymeric chains using two click reactions, namely thiol-ene chemistry and the azide/alkyne cycloaddition reaction. While thiol-ene chemistry was used to attach azide groups onto the surface of microspheres, alkyne-end-functional polymers were then reacted with the azide-modified surface via Huisgen 1,3-dipolar cycloaddition. More recently, we reported an alternative method to obtain directly azide-modified surfaces through hydrobromination followed by azidation reactions.[40]

In order to demonstrate the versatility of the described functionalization process, PDVB microspheres were subsequently coupled with a small fluorescent molecule – an alkyne-modified pyrene – by the copper-catalyzed Huisgen 1–3 dipolar cycloaddition click reaction.

In this paper, PDVB cross-linked core microspheres carrying hydrophilic and/or hydrophobic chains onto the surface are synthesized, characterized and used as the support matrix for the reversible immobilization of *Agaricus bisporus* laccase via adsorption. Based on the support structures, the immobilizing behavior is investigated. The system parameters, such as the adsorption conditions (i.e., enzyme concentration, medium pH), as well as desorption, are studied and evaluated in terms of enzymatic activity and adsorption capacity.

Experimental Part

Materials

DVB (55% mixture of isomers, DVB55, technical grade, Aldrich) was used as received. Acetonitrile (99%, Aldrich) was distilled over CaH_2 before use. AIBN (Fluka) was recrystallized from methanol. *N,N,N',N'',N''*-Pentamethyldiethylenetriamine (PMDETA, 99%, Aldrich) was distilled before use. Methyl methacrylate (MMA, 99%, Aldrich) and *tert.*-butyl acrylate (*t*BA, 99%, Aldrich) were passed through a column containing basic alumina. Dichloromethane (CH_2Cl_2, 99%, Lab-Scan) was distilled over P_2O_5. *N,N'*-Dicyclohexylcarbodiimide (DCC, 99%, Aldrich), 4-dimethylaminopyridine (DMAP, 99%, Acros), 4-pentynoic acid (99%, Aldrich), triethylamine (TEA, 99.5%, Fluka), ethylene glycol (95%, Aldrich), 2-bromoisobutyryl bromide (97%, Aldrich), propargyl alcohol (99%, Aldrich), poly(ethylene glycol) monomethyl ether (Me-PEG, $\overline{M}_n$ = 2 000 and 5 000, Fluka), *N,N*-dimethylformamide (DMF, ≥99%, Aldrich), sulfuric acid (95–97%, Fluka), potassium bromide (≥99.5%, Merck), sodium azide (NaN_3, ≥99%, Merck), sodium chromate (Merck), silver nitrate (Merck), CuBr (98%, Acros), methanol (99%, Riedel-de Haën), diethyl ether (98%, Carlo-Erba) and tetrahydrofuran (THF, 99% Lab-Scan) were used as received.

2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was supplied by Sigma Chemical Co (St. Louis, USA). Crystallized and lyophilized laccase (Lac; Benzenediol: Oxygen oxidoreductase; from *Agaricus bisporus*, E.C 1.10.3.2, powder, deep brown, 8.85 $U \cdot mg^{-1}$) was obtained from Fluka (Steinheim, Germany).

Synthesis of PDVB Microspheres

The PDVB microspheres were prepared by the precipitation-polymerization technique, as described in the literature.[8] For this purpose, AIBN (0.2 g, 1.22 mmol, 4 wt.-% relative to DVB55) was added to a solution of DVB55 (11 mL, 76.8 mmol, 4 vol.-% relative to the total volume) and 274 mL of acetonitrile, in a dry, 500 mL three-necked flask equipped with a mechanical stirrer and a nitrogen inlet. The flask was placed in a thermostatted oil bath and the temperature was adjusted to 70 °C. The nitrogen flow was stopped and the reaction was conducted for 48 h at this temperature under continuous stirring (32 rpm). The reaction content was cooled to room temperature and precipitated polymer was filtered and washed with THF (20 mL), acetone (20 mL) and methanol (20 mL). The product was dried at 45 °C overnight under vacuum. The yield was 3 g (30%).

Fourier-transform infrared (FTIR) spectroscopy: $\nu = 3\,014$–$2\,915$, 1 704, 1 679, 1 628, 1 601, 1 509, 1 484, 1 445, 1 207, 1 116, 1 117, 1 060, 1 015, 988, 900, 834, 795, 708 cm^{-1}.

Hydrobromination of PDVB Microspheres

Hydrobromination was performed according to a procedure described in the literature.[41] PDVB microspheres (2 g) were suspended using 80 mL of n-heptane in a 250 mL round-bottom flask; dibenzoyl peroxide (0.18 g, 0.74 mmol) was added to the mixture under stirring with a magnetic stirrer. Hydrogen bromide (HBr), generated by drop-wise addition of concentrated sulfuric acid (15 mL, 0.28 mol) to solid KBr (59.5 g, 0.50 mol) in a separate vessel, was introduced to the mixture by means of a delivery tube. After 8 h, the generation of HBr was stopped and the stirring was maintained for another 2 h. The suspension mixture was filtered and the product was purified by washing with dichloromethane, methanol and diethyl ether before drying in a vacuum oven at 40 °C overnight. The dried product was weighed as 2.4 g.

The amount of bromine in the particles was determined by titration; the brominated particles (0.1 g) were mixed with magnesium oxide (1 g, 25 mmol) and sodium carbonate (0.5 g, 4.71 mmol) and fused at 550 °C for 10 h, as described in the literature.[42] Sodium bromide in the cooled mixture was transferred into a 100 mL volumetric flask by appropriate dilutions and washings and slightly acidified (pH = 5) by adding nitric acid (65%). This solution was titrated with $AgNO_3$ in the presence of sodium chromate as a color indicator. This estimation revealed a bromine content of 1.46 $mmol \cdot g^{-1}$.

FTIR: $\nu = 3\,018$–$2\,920$, 1 676, 1 599, 1 484, 1 443, 1 372, 1 210, 1 178, 1 113, 1 061, 1 043, 1 016, 966, 891, 829, 794 cm^{-1}.

Azidation of PDVB Microspheres (PDVB-N_3)

The bromoethyl group on the PDVB microspheres was converted into an ethyl azide function by condensation with NaN_3. For this purpose, PDVB microspheres (1 g) were suspended in 20 mL of DMF and NaN_3 (0.5 g, 7.7 mmol) was added to the mixture. Then, 0.1 mL of distilled water was added to enhance the solubility of the sodium azide. The flask was closed and covered with aluminum foil to protect from light exposure and shaken for 24 h at room temperature. The bead product was isolated by filtration and successive washings with water (20 × 2 mL), methanol (20 mL) and diethyl ether (20 mL) and was dried overnight under vacuum at room temperature. On the basis of the bromine content and the quantitative azidation reaction, the amount of the azide group was estimated to be 1.46 mmol N_3 units per g.

FTIR: $\nu = 3\,018$–$2\,920$, 2 870, 2 099, 1 702, 1 603, 1 508, 1 484, 1 374, 1 243, 1 174, 1 061, 893, 829, 794, 703 cm^{-1}.

Synthesis of the Alkyne-Functional ATRP Initiator

Initiator was synthesized by drop-wise addition of 2-bromoisobutyryl bromide (1.5 mL, 12 mmol) to propargyl alcohol (0.68 mL, 11.5 mmol) and TEA (1.65 mL, 11.9 mmol) solution in diethyl ether (50 mL) at 0 °C under a nitrogen atmosphere. After addition, the reaction mixture was stirred overnight at room temperature. The reaction mixture, extracted with water and organic phase, was dried with Na_2SO_4. The solution was concentrated and the crude product was purified by column chromatography over silica gel, eluting with hexane/ethyl acetate (10/1) to give the product as a colorless liquid (yield = 1.9 g, 85%).

^1H NMR ($CDCl_3$): $\delta = 1.90$ (s, 6H,), 2.47 (t, 1H,), 4.71 (s, 2H,)

Preparation of the Alkyne-Functional Poly(ethylene glycol) (Alkyne-PEG-2 000 or Alkyne-PEG-5 000)

Poly(ethylene glycol) (PEG, $\overline{M}_n = 2\,000\ g \cdot mol^{-1}$, 3.0 g, 1.5 mmol) was dissolved in 25 mL of CH_2Cl_2. 4-Pentynoic acid (0.22 g, 2.25 mmol) and DMAP (0.18 g, 1.5 mmol) were successively added to the reaction mixture. After stirring for 5 min at room temperature, a solution of DCC (0.46 g, 2.25 mmol) in 15 mL of CH_2Cl_2 was added to the reaction mixture and stirred overnight at room temperature. After filtration of the salt, the solution was concentrated and product was purified by column chromatography over silica gel, eluting with a CH_2Cl_2/ethyl acetate mixture (1:10) and then with CH_2Cl_2/MeOH (10:1). Finally, a concentrated solution of alkyne-PEG was precipitated in diethyl ether and filtered (yield = 2.81 g, $\overline{M}_n$, GPC = 2 400, $\overline{M}_w/\overline{M}_n = 1.08$).

^1H NMR ($CDCl_3$): $\delta = 4.23$ (2H, CO–O–CH_2–), 3.62 (4H, O–CH_2–CH_2–O–), 3.35 (s, 3H, CH_2–CH_2–O–CH_3), 2.60–2.28 (m, 4H, CH–CH_2–CH_2–CO–O), 1.95 (CH–CH_2–CH_2–CO–O). FTIR: $\nu = 3\,265$, 2 883, 2 740, 2 162, 1 736, 1 556, 1 466, 1 359, 1 1341, 1 279, 1 240, 1 146, 1 103, 1 060, 959, 841 cm^{-1}.

Preparation of the Alkyne-Functional Poly(methyl methacrylate) (Alkyne-PMMA)

CuCl (66.8 mg, 0.67 mmol), PMDETA (97.2 μL, 0.67 mmol), alkyne-functional initiator (96 mg, 0.46 mmol), methyl methacrylate (5 mL, 46.6 mmol) and 5 mL of toluene as a solvent were placed in a Schlenk tube. Three freeze-pump-thaw cycles were performed and the tube was stirred in an oil bath at 80 °C for 20 min. At the end of the reaction, the mixture was diluted with THF and the copper complex was removed by passing through a neutral alumina column. The diluted mixture was precipitated in hexane and the solid was collected after filtration and dried at room temperature in a vacuum overnight (35%, $\overline{M}_{n,theo} = 3\,800\ g \cdot mol^{-1}$, $\overline{M}_{n,GPC} = 5\,400$, $\overline{M}_w/\overline{M}_n = 1.20$).

^1H NMR ($CDCl_3$): $\delta = 4.65$ (2H, CH–CH_2–), 3.58 (3H, CO–O–CH_3), 2.46 (1H, CH–CH_2), 2.05–0.60 (aliphatic protons). FTIR: $\nu = 3\,449$, 2 997, 2 952, 1 729, 1 483, 1 448, 1 387, 1 269, 1 241, 1 190, 1 148, 1 066, 989, 911, 841, 750, 668 cm^{-1}.

Synthesis of the Alkyne-Functional Poly(*tert*-butyl acrylate) (Alkyne-P*t*BA)

A Schlenk tube was charged with CuBr (51.1 mg, 0.35 mmol), PMDETA (74.3 μL 0.35 mmol), alkyne-functional initiator (73.1 mg, 0.35 mmol), and *tert*.-butyl acrylate (5 mL, 34.1 mmol). Three freeze-pump-thaw cycles were performed and the tube was stirred in an oil bath at 80 °C for 40 min. After the given time, the mixture was diluted with THF. The copper complex was then removed by passing through a neutral alumina column and THF was removed by rotary evaporation. The mixture was precipitated in cold methanol/water (80/20 v/v). After decanting, the polymer was dissolved in CH_2Cl_2, extracted with water and the water phase was

again extracted with CH_2Cl_2. The combined organic phase was dried over Na_2SO_4. Finally, the organic phase was evaporated to give alkyne-functional poly(*tert.*-butyl acrylate). The polymer was dried for 24 h in a vacuum oven (32%, $\overline{M}_{n,theo} = 4\ 100\ g \cdot mol^{-1}$, $\overline{M}_n$, GPC = 5 130, $\overline{M}_w/\overline{M}_n = 1.30$).

^{1}H NMR ($CDCl_3$): $\delta = 4.05$ (1H, *CH*–Br), 2.45 (1H, *CH*–CH_2), 2.20 [*CH*–CO–O–C$(CH_3)_3$]. FTIR: $\nu = 3\,444, 2\,975, 2\,929, 2\,129, 1\,723, 1\,476, 1\,448, 1\,392, 1\,366, 1\,257, 1\,144, 1\,019, 844, 793, 751, 662\ cm^{-1}$.

PEG-Grafted PDVB Microspheres via Click Reaction (PDVB-*g*-PEG)

PDVB microspheres (100 mg containing 1.45×10^{-4} mol azide groups) were suspended in 4 mL of DMF in a Schlenk tube. Alkyne-PEG-2 000 (0.7 g, 2.92×10^{-4} mol), CuBr (20.94 mg, 1.45×10^{-4} mol), and PMDETA (30.4 μL, 1.45×10^{-4} mol) were added and the reaction mixture was degassed by three freeze-pump-thaw cycles and left in vacuo. The mixture was stirred at 40 °C for 48 h. The solid was separated from the mixture by centrifugation. The collected solid was redispersed in DMF and separated by centrifugation. This purification cycle was repeated three times for dilute acidic methanol solution and THF. After purification, the resulting products were dried overnight under vacuum, and a sample of PEG-grafted PDVB microspheres (102 mg) was obtained.

FTIR: $\nu = 2\,983–2\,864, 2\,100, 1\,727, 1\,705, 1\,603, 1\,516, 1\,486, 1\,446, 1\,373, 1\,242, 1\,087, 1\,056, 1\,017, 892, 829\ cm^{-1}$.

PEG-and-PMMA-Grafted PDVB Microspheres via Click Reaction (PDVB-*g*-PEG/PMMA)

PDVB microspheres (50 mg containing 0.73×10^{-4} mol azide groups) were suspended in 5 mL of DMF in a Schlenk tube. Alkyne-PEG-5 000 (0.79 g, 1.46×10^{-4} mol), alkyne-PMMA (0.78 g, 1.46×10^{-4} mol), CuBr (10.45 mg, 0.73×10^{-4} mol), and PMDETA (15.2 μL, 0.73×10^{-4} mol) were added and the reaction mixture was degassed by three freeze-pump-thaw cycles and left in vacuo. The mixture was stirred at 40 °C for 48 h. The solid was separated from the mixture by centrifugation. The collected solid was redispersed in DMF and separated by centrifugation. This purification cycle was repeated three times for dilute acidic methanol solution and THF. After purification, the resulting products were dried overnight under vacuum, and a sample of PEG-and-PMMA-grafted PDVB microspheres (60 mg) was obtained.

FTIR: $\nu = 3\,046–2\,920, 2\,097, 1\,728, 1\,603, 1\,510, 1\,484, 1\,447, 1\,387, 1\,079, 968, 893, 826, 794, 707\ cm^{-1}$.

PEG-and-P*t*BA-Grafted PDVB Microspheres via Click Reaction (PDVB-*g*-PEG/P*t*BA)

PDVB microspheres (50 mg containing 0.73×10^{-4} mol azide groups) were suspended in 5 mL of DMF in a Schlenk tube. Alkyne-PEG-5 000 (0.79 g, 1.46×10^{-4} mol), alkyne-P*t*BA (0.73 g, 1.46×10^{-4} mol), CuBr (10.5 mg, 0.73×10^{-4} mol), and PMDETA (15.2 μL, 0.75×10^{-4} mol) were added and the reaction mixture was degassed by three freeze-pump-thaw cycles and left in vacuo. The mixture was stirred at 40 °C for 48 h. The solid was separated from the mixture by centrifugation. The collected solid was redispersed in DMF and separated by centrifugation. This purification cycle was repeated three times for dilute acidic methanol solution and THF. After purification, the resulting products were dried overnight under vacuum, and a sample of PEG-and-P*t*BA-grafted PDVB microspheres (70 mg) was obtained.

FTIR: $\nu = 3\,026–2\,923, 2\,099, 1\,728, 1\,666, 1\,610, 1\,508, 1\,486, 1\,445, 1\,368, 1\,150, 1\,085, 968, 893, 826, 794, 707\ cm^{-1}$.

Characterization

Gel-permeation chromatography (GPC) measurements were obtained using a Viscotek GPCmax Autosampler system consisting of a pump, a Viscotek UV detector and a Viscotek differential refractive-index (RI) detector. Three ViscoGEL GPC columns ($G2000H_{HR}$, $G3000H_{HR}$ and $G4000H_{HR}$), (7.8 mm internal diameter, 300 mm length) were used in series. The effective molecular-weight ranges were 456–42 800, 1 050–107 000, and 10 200–2 890 000, respectively. THF was used as the eluent at a flow rate of $1.0\ mL \cdot min^{-1}$ at 30 °C. Both detectors were calibrated using PS standards, having a narrow molecular-weight distribution. The data were analyzed using the Viscotek OmniSEC Omni-01 software. The molecular weights were calculated with the aid of polystyrene standards. The FTIR spectra were recorded on a Perkin-Elmer FTIR Spectrum One B spectrometer. Thermogravimetric analysis (TGA) was performed on a Perkin-Elmer Diamond TA/TGA instrument at a heating rate of 10 °C min^{-1} under nitrogen flow.

Enzyme Adsorption and Desorption Experiments

Adsorption of the laccase enzyme on the different types of microspheres was studied at various pH in sodium acetate (1 mL, 0.05 M, pH = 3.5–5.0). The initial concentration of the enzyme was $10\ mg \cdot mL^{-1}$ in each corresponding buffer. The adsorption experiments were conducted for 120 min at 25 °C in an orbital shaker. The equilibrium adsorption time was determined to be 120 min by preliminary experiments. The amount of laccase adsorbed on the three types of microspheres was determined by following the initial and final concentrations of the protein within the adsorption medium using Coomassie Brilliant Blue with crystalline bovine serum albumin (BSA) as the standard.[43] The experiments were performed in replicates of three and the samples were analyzed in replicates of three as well. For each set of data present, standard statistical methods were used to determine the mean values and standard deviations.

Laccase was removed from the microspheres (after adsorption) by washing in 10% PEG and 1.0 M $(NH_4)_2SO_4$ (desorbing buffer) for 90 min at 25 °C.[44] The desorption ratio of the enzyme was calculated by using the following expression:

$$\text{Desorption ratio} = (\text{enzyme released/adsorbed enzyme on microspheres}) \times 100 \quad (1)$$

Activity Assays

The laccase activity was assessed by measurement of the enzyme oxidation of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) at 427 nm ($\varepsilon = 3.6 \times 10^4\ cm^{-1} \cdot L \cdot mol^{-1}$).[45] The reaction mixture

contained either free or immobilized enzyme in 300 μL of 10^{-3} M ABTS and 0.1 M Na acetate buffer (pH = 4.5). 1.0 Unit (U) of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol ABTS in 1 min. The specific activity is expressed as U per mg of protein per g of support matrix.

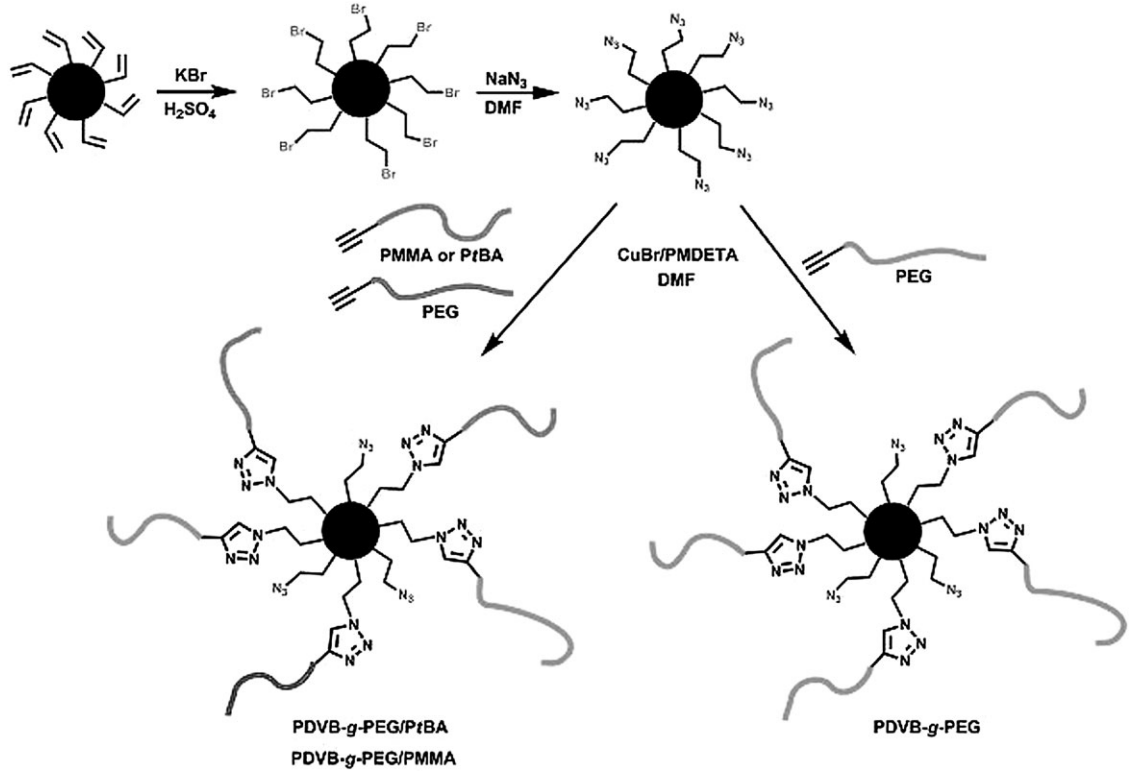
Zeta-Potential Analysis

A Zeta-Meter 3.0+ (with Zeiss DR microscope, GT-2 type quartz cell, molybdenum-cylinder anode, and platinum-rod cathode electrode) was used for the zeta-potential analysis using sodium acetate buffer (pH = 4.5, 0.1 M) as the environment. The microspheres (0.5 mg · mL^{-1}) were suspended in the buffer by stirring overnight. In electrophoresis, the particles are moved by applying an electric field across the system. The microspheres were timed for both directions of the applied electric field. The value of the zeta potential assigned to the dispersions was the average of the data obtained from 6 experiments. The applied voltage during the measurements was varied in the range 20–30 mV.[46] The zeta potential of the microsphere suspensions was estimated from the measured electrophoretic mobilities by employing the Smoluchowski equation. The electrokinetic charge densities were also calculated according to Saka et al.[47]

Results and Discussion

The overall process, as presented in Scheme 1, is of special concern in the preparation of microspheres containing hydrophilic and/or hydrophobic polymer chains via the click reaction. For this purpose, the residual double bonds of the PDVB microspheres prepared by the precipitation-polymerization technique were activated by hydrobromination and the ethyl bromide groups formed in this way were then converted into azide functions by condensation with NaN_3 in DMF solution. As demonstrated in the previous article[40] for the incorporation of the model compound, the method has the advantage of preparing azide functions directly from the microspheres by two, simple organic reactions. Moreover, the ability of changing the surface properties of the spheres through a wide range of alkyne-functional low-molar-mass compounds and polymers accessible through simple synthetic methods will likely benefit, particularly in biological applications. In the present work, the required alkyne-functional polymers were independently synthesized using ATRP and esterification techniques. The results and conditions for the preparation of the alkyne-functional polymers are summarized in Table 1.

The click reaction was performed using a Cu(I) catalyst in DMF at 40 °C. After the coupling reaction, the polymer-grafted PDVB microspheres could be easily collected by centrifugation. The catalyst and the excess polymers were removed by washing with DMF, dilute acidic methanol and THF. The polymer-grafted microspheres were characterized by IR spectroscopy (Figure 1), where a reduction of the azide



Scheme 1. Preparation of microspheres containing hydrophilic and/or hydrophobic polymer chains by hydrobromination and click reactions.

Table 1. Preparation of the alkyne-functional polymers.

Polymer	Method	Time	Conversion[d)]	$\overline{M}_{n,theo}$[e)]	$\overline{M}_n$, GPC[f)]	$\overline{M}_w/\overline{M}_n$[f)]
		min	%	g · mol^{-1}	g · mol^{-1}	
alkyne-P*t*BA[a)]	ATRP	60	32	4 100	5130	1.30
alkyne-PMMA[b)]	ATRP	20	35	3 800	5400	1.20
alkyne-PEG1[c)]	esterification	overnight	75	–	5430	1.02
alkyne-PEG2[c)]	esterification	overnight	93	–	2440	1.02

[a)] $[M]_0:[I]_0:[CuBr]_0:[PMDETA]_0 = 100:1:1:1$, $T = 80\,^{\circ}C$; [b)] In Toluene, $[M]_0:[I]_0$: $[CuCl]_0:[PMDETA]_0 = 100:1:1:1$, $T = 80\,^{\circ}C$; [c)] Polymers were synthesized by the esterification reaction between 4-pentynoic acid and poly(ethylene glycol) monomethyl ether at room temperature; [d)] Determined gravimetrically; [e)] Calculated using $\overline{M}_{n,theo} = ([M]_0/[I]_0 \times \%\ \text{conversion} \times 100 + \overline{M}_{w,I})$; [f)] Determined by GPC based on polystyrene standards.

vibration peak at 2 099 cm^{-1} was observed, together with a concomitant increase in the peak at 1 085 cm^{-1}, corresponding to the ether linkage of the PEG grafts. The absorptions of the carbonyl groups of the hydrophobic segments were also noted at 1 728 cm^{-1}. Comparison of the peak areas at 2 099 cm^{-1} before and after the click reaction clearly indicates that most of the azide groups were successfully reacted.

TGA analysis also confirmed the presence of grafted chains on the surfaces of the microspheres (Figure 2). The azidated microspheres exhibited an additional 13% weight loss, which corresponds to the decomposition of the azide group. After the click reaction, the weight loss in that region was decreased, indicating the reaction between the alkyne-functional polymers and the azide groups. The grafting efficiency was calculated by using a weight loss of 17, 20 and 27% for the PDVB-*g*-PEG, PDVB-*g*-PEG/P*t*BA, and PDVB-*g*-PEG/PMMA, respectively. Other evidence obtained from the TGA measurements was the char yield, calculated as 19.4, 30.1, and 31.9%, which changed proportionally with the grafting efficiency.

Biomolecule Immobilization

In this part, PDVB-*g*-PEG, PDVB-*g*-PEG/P*t*BA and PDVB-*g*-PEG/PMMA were investigated as the platforms for reversible biomolecule immobilization via adsorption. *A. bisporus* laccase was used as a model protein to examine the adsorption and desorption properties of the modified microspheres. Finally, these microspheres were evaluated in terms of their protein-adsorption capacities, as well as the activity retention in the immobilized form.

Since pH influences the stability and conformational structure of proteins, this parameter should be controlled during adsorption of enzymes to obtain reproducible results. Figure 3 shows the amount of enzyme adsorbed

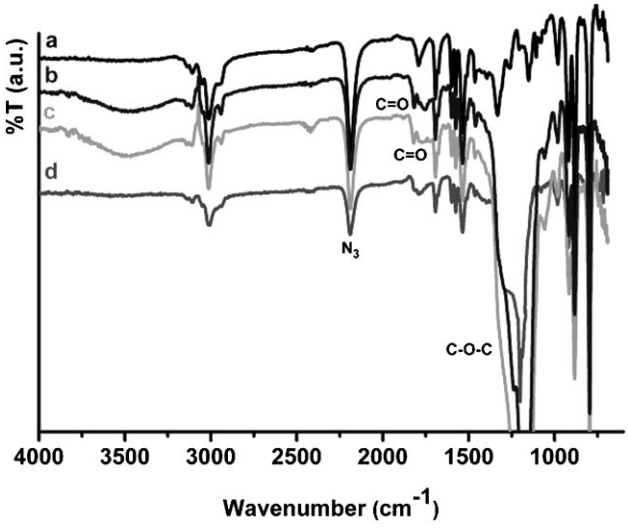


Figure 1. FTIR spectra of (a) PDVB-N_3 microspheres, (b) PDVB-*g*-PEG/P*t*BA microspheres, (c) PDVB-*g*-PEG/PMMA microspheres and (d) PDVB-*g*-PEG microspheres.

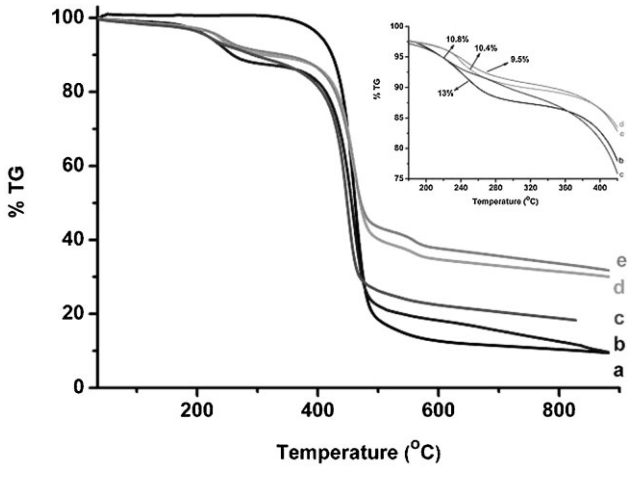


Figure 2. TGA traces for (a) PDVB microspheres, (b) PDVB-N_3 microspheres, (b) PDVB-*g*-PEG microspheres, (c) PDVB-*g*-PEG/P*t*BA microspheres, and (d) PDVB-*g*-PEG/PMMA microspheres.

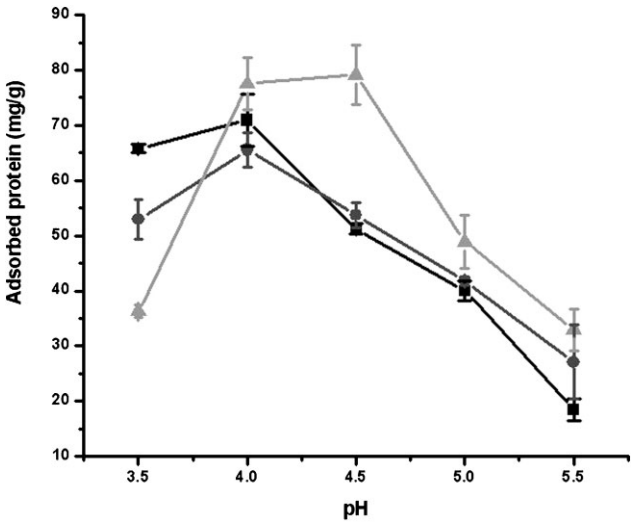


Figure 3. Effect of pH on the adsorptive amount of the microspheres [adsorption buffer; 5×10^{-2} M acetate buffer; adsorption time = 2 h; 25 °C; ■ (PDVB-*g*-PEG), ● (PDVB-*g*-PEG/P*t*BA), ▲ (PDVB-*g*-PEG/PMMA); the error bars show the standard deviations (SDs)].

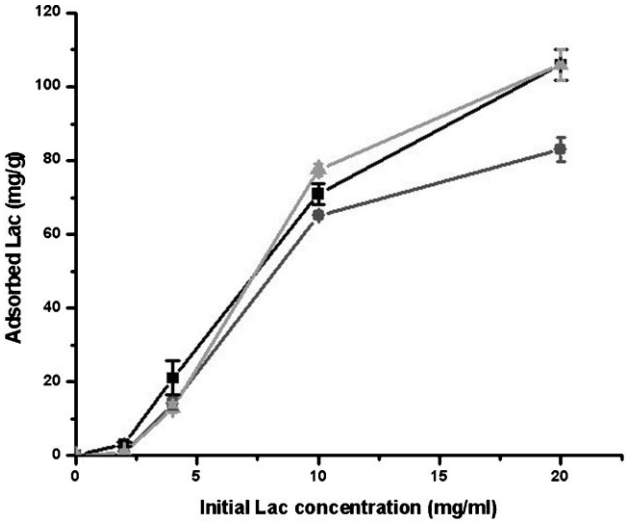


Figure 4. Effect of enzyme amount on the adsorptive amount of the microspheres [adsorption buffer; pH = 4.0, 50×10^{-3} M acetate buffer; adsorption time = 2 h; 25 °C; ■ (PDVB-*g*-PEG), ● (PDVB-*g*-PEG/P*t*BA), ▲ (PDVB-*g*-PEG/PMMA); the error bars show the SDs].

onto the microspheres at different pH values. Close to the isoelectric point (pH_i) of the *A. bisporus* laccase, at a pH of 4.0, the adsorption amount exhibited a maximum, which is a rather-general phenomenon in protein adsorption. Since the biomolecules do not have a net charge at the pI, the electrostatic repulsion between the adsorbed protein molecules is at a minimum. Hence, the proteins could attain closer packing on the surface of the microspheres than on those with a net charge. Another reason for the maximum might be due to a higher structural stability and therefore a smaller tendency to spread at the interface of the microspheres at pHs closer to their isoelectric regions, as also reported for the other systems.[48] Figure 4 presents the relation between the enzyme concentration and the adsorptive amount. As can be seen, with the increase of enzyme concentration, the adsorption amount increases correspondingly up to 10 mg · mL^{-1} and a less-significant increase was obtained at 20 mg · mL^{-1}. Comparison of the adsorbed amounts per g of support at pH = 4.0 shows that the PDVB-*g*-PEG/PMMA surface attracts more enzyme molecules (77.5 mg per g of support) compared to PDVB-*g*-PEG (71 mg per g of support) and PDVB-*g*-PEG/P*t*BA, (65 mg per g of support). The higher adsorption capacity of PDVB-*g*-PEG/PMMA can be explained by the presence of both PEG and PMMA on the surface, which causes both hydrophobic and hydrophilic interactions during the protein adsorption, because of the dual functionality, while the PDVB-*g*-PEG surface attracts protein molecules only by hydrophilic interactions. In contrast, among all of the matrices, the lowest protein adsorption, to PDVB-*g*-PEG/P*t*BA, might be due to the branched structure of the P*t*BA, which may prevent a good contact between the matrix and the biomolecule, and this could affect the enzyme-loading capacity, as well as the biocatalytic activity.

The ability of immobilizing proteins with high binding capacities on surfaces while maintaining their activity is one of the most-critical parameters for biotechnological applications.[49] The specific activities of the immobilized laccase are shown in Table 2 after adsorption of 10 mg · mL^{-1} and 20 mg · mL^{-1} enzymes. According to the data, higher specific activities were observed for all supports after the 10 mg · mL^{-1} enzyme-loading process. The performance of the adsorptive immobilized enzyme is largely determined by the chemical and physical nature of the carriers. The density of the binding functionality is one of the important facts in non-covalent binding: it affects the microenvironment of the carrier. The conformational flexibility of the enzyme is mainly related to the number of links to the enzymes in the microenvironment and the loading capacity depends on the number of binding sites.[50] In our case, the highest adsorption capacity was obtained with PDVB-*g*-PEG/PMMA; in contrast, replacing the PMMA with P*t*BA in the structure led to a reduced enzyme loading and activity because of the improper matrix structure, which prevented the protection of the flexible biomolecule conformation for efficient immobilization. The highest specific activity was observed using PDVB-*g*-PEG. This can be explained by the fact that the hydrophobic tails on the PDVB-*g*-PEG/PMMA and PDVB-*g*-PEG/P*t*BA microspheres could interact with each other via the hydrophobic forces of the polar reaction medium (acetate buffer). This could create network-like assemblies that force the enzyme to be locked, probably in an improper conformation, which could cause a drop in the activity. This is in contrast to PDVB-*g*-

Table 2. Effect of enzyme loading on the biocatalytic activity of immobilized enzymes.

Microspheres	Initial laccase amount	Specific activity
	$mg \cdot mL^{-1}$	$U \cdot (mg\ protein)^{-1} \cdot (g\ support)^{-1}$
PDVB-*g*-PEG	10	1.14
PDVB-*g*-PEG	20	0.76
PDVB-*g*-PEG/PMMA	10	0.46
PDVB-*g*-PEG/PMMA	20	0.39
PDVB-*g*-PEG/P*t*BA	10	0.26
PDVB-*g*-PEG/P*t*BA	20	0.21

PEG in which a mild microenvironment is available for the enzyme because of rather-weak hydrophilic forces. Additionally, PEGs and PEGylated surfaces are of extreme importance in the field of biomedical engineering due to their ability to control biomolecular interactions with the device surface. They have been used to prevent biofouling in bio-microsystems, tissue-engineering applications, drug delivery, and cell patterning.[51] It can be stated that all of these properties might contribute to provide a biocompatible surface, and use of PDVB-*g*-PEG could be advantageous with regard to the biocatalytic activity.

PEG (10%) and $(NH_4)_2SO_4$ (1 M) were used as desorption agents to remove the proteins from the microspheres. Approximately, 100% desorption was observed for all of the matrices when PEG and $(NH_4)_2SO_4$ were applied together over 90 min. It is clear that to achieve almost complete desorption is important for matrix regeneration, due to the possible reusability, especially in industrial applications. It was also observed that PEG alone desorbed 76% whereas $(NH_4)_2SO_4$ desorbed 95% of the adsorbed laccase for the PDVB-*g*-PEG/P*t*BA. However, the amount of laccase desorbed by PEG from the PDVB-*g*-PEG/PMMA was 80% and it was 67% by $(NH_4)_2SO_4$. It can be stated that, in the case of the PDVB-*g*-PEG/PMMA, the hydrophobic interactions were more effective in the laccase adsorption compared to PDVB-*g*-PEG/P*t*BA.[42] Hence, PDVB-*g*-PEG/PMMA seems to be a more-efficient platform for attracting biomolecules via hydrophobic forces, which is important for specific protein-adsorption techniques, especially useful for biomolecules with a hydrophobic nature. On the other hand, due to the highest protein capacity, this microsphere could be a promising protein-purification or removal medium by further optimization, adjusting the ratio of the surface functionalities according to the desired systems.

The zeta potential (ZP) is an indicator of the surface-charge properties of a colloid or a particle in solution and varies depending on the surface potential and the thickness of the electric double layer. The degree of repulsion between the charged particles in a dispersion is indicated by the zeta potential. A high zeta potential shows stability of small particles and resistance to aggregation. To further understand the interaction of the protein molecules (laccase) and microspheres, their electrical properties should be known. Table 3 shows the ZP and electrokinetic charge densities for

Table 3. Zeta potential and electrokinetic charge densities for the microspheres before and after enzyme loading and desorption in the presence of PEG and $(NH_4)_2SO_4$.

Polymer	Method	Zeta potential[a)]	Electrokinetic charge density
		mV	$C \cdot m^{-2}$
PDVB-*g*-PEG	carrier	-54.00 ± 1.585	-47.00×10^{-3}
PDVB-*g*-PEG	enzyme loading[b)]	-77.89 ± 1.36	-81.20×10^{-3}
PDVB-*g*-PEG	desorption	-58.79 ± 0.54	-52.86×10^{-3}
PDVB-*g*-PEG/P*t*BA	carrier	-88.64 ± 2.66	-101.84×10^{-3}
PDVB-*g*-PEG/P*t*BA	enzyme loading[b)]	-101.02 ± 2.99	-131.23×10^{-3}
PDVB-*g*-PEG/P*t*BA	desorption	-92.24 ± 3.25	-109.71×10^{-3}
PDVB-*g*-PEG/PMMA	carrier	-88.40 ± 3.09	-101.34×10^{-3}
PDVB-*g*-PEG/PMMA	enzyme loading[b)]	-101.40 ± 3.19	-132.24×10^{-3}
PDVB-*g*-PEG/PMMA	desorption	-90.17 ± 1.98	-105.12×10^{-3}

[a)]All measurements were repeated 5 or 6 times and the data are expressed as mean ± SD; [b)]10 mg enzyme was loaded to each carrier.

the microspheres before and after enzyme loading and desorption in the presence of PEG and $(NH_4)_2SO_4$. As can be seen from the table, the ZP values registered as negative and electrically stabilized. After enzyme loading, as a result of adsorption, the ZPs were changed to more-negative values, indicating that the loaded enzyme was negatively charged in the operational conditions. It was also found that desorption of the enzyme resulted in a decrease in the ZP. When the PDVB-*g*-PEG, PDVB-*g*-PEG/P*t*BA, and PDVB-*g*-PEG/PMMA carriers were compared with each other, the PDVB-*g*-PEG carrier was shown to have a less-negative zeta-potential value because of the lower negative charge on its surface. This phenomenon was also verified by the calculation of the electrokinetic charge densities. Similar ZP values, after biomolecule desorption, could be a good indication of an almost-complete matrix regeneration, which is important for matrix recovery.

Conclusion

The synthesis of materials with controlled structures and architectures continues to be a focus of considerable current research. Amphiphilic copolymers offer good opportunities for controlling interfacial properties, and since they self-assemble in hydrophilic/hydrophobic environments such as at the air/water (A/W) interface to form regular arrangements of 2D surface micelles, they are particularly interesting as precursors for the synthesis of well-defined patterns.[52] On the other hand, progress in bioimmobilization has resulted in a revolution of the use of biomolecules for various applications in separation, catalysis, and sensors, that typically rely to large part on the successful immobilization of the intact biomolecules onto or within a suitable host.[53] In this paper, PDVB cross-linked core microspheres having hydrophilic and/or hydrophobic tails onto the surface were synthesized, characterized and used as the support matrix for the reversible immobilization of *Agaricus bisporus* laccase via adsorption. The system parameters such as the adsorption conditions (i.e., enzyme concentration, medium pH) as well as desorption were studied and evaluated with regard to the enzymatic activity and loading capacity. It can be concluded that PDVB-*g*-PEG/PMMA, due to the dual functionalities that caused higher protein loading, could be a promising platform for the reversible protein adsorption, which is important in various biotechnological applications. On the other hand, PDVB-*g*-PEG exhibited a better performance in terms of keeping the biocatalytic activity by providing mild conditions that allowed the biomolecules to be kept in their active and flexible conformations without any diffusion problems for the substrate. Therefore, it could be possible to use this microsphere for the reversible immobilization of various biocatalytic proteins that may be good candidates for industrial applications.

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