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Trypsin Immobilized on Magnetic Beads via Click Chemistry: Fast Proteolysis of Proteins in a Microbioreactor for MALDI-ToF-MS Peptide Analysis

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ABSTRACT: In the present work, magnetic poly(2-hydroxyethyl methacrylate–ethyleneglycol dimethacrylate) (mp(HEMA-EGDMA)) beads were prepared by suspension polymerization. The beads were grafted with poly(hydroxylpropyl methacrylate) (pHPMA) and used for immobilization of trypsin via click chemistry. The p(HMPA) units in the brushes made the fibrous polymer hydrophilic. Synthesized beads were characterized by swelling studies, Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The bromo groups of the grafted polymer were modified into azide groups, and the enzyme was alkyne-functionalized for conjugation with azide-modified magnetic beads via click reaction. The kinetic constants (K_m and V_{max}) were determined by measuring initial reaction rates using the artificial chromogenic substrate *N*-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA). The optimal pH value for the immobilized enzyme was slightly higher compared to free enzyme. The immobilized trypsin possessed much higher thermal stability than the free enzyme. Finally, the immobilized enzyme was placed in a microbioreactor for efficient proteolysis of model proteins, namely, bovine serum albumin (BSA), lysozyme, and cytochrome *c*. The immobilized trypsin in the microbioreactor provided a promising platform for model protein identification and was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) analysis.

INTRODUCTION

Enzyme immobilization is one of the initial key issues for many applications such as microarrays, microbioreactors, and biosensors.^{1–5} However, the final success depends on the selection of immobilization methods and support materials.^{5,6} Recently, polymer grafted materials represent an attractive support family for use in enzyme technology. Controlled synthesis of graft polymers on the beaded support could be realized by using atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization, and reversible addition–fragmentation chain-transfer polymerization (RAFT).^{7–9} The former method is attractive due to its mild reaction conditions, tolerance to impurities, and use of readily available initiators and monomers.^{10–12} Moreover, surface initiated-ATRP can result in minimal homopolymerization in the reaction medium and allow control over polymer thickness by variation of polymerization time.^{12–14}

Magnetic support materials have additional importance for enzyme immobilization and for use in continuous bioassay systems and microbioreactor configurations.^{15,16} However, magnetic beads with grafted fibrous polymers allow the immobilization of large amounts of enzyme on a protective hydrophilic polymer layer. Also, magnetic separation is relatively simple and economical, requiring a simple apparatus and little space compared to gravity separation systems.

Magnetic support with a biocatalyst can be isolated from reaction medium by a magnetic device and simply retained in the microreactor system after removal of the magnetic field. Recently, there has been increased interest in the use of magnetic supports in bioanalysis applications.^{1,17,18}

Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system, where it hydrolyzes proteins. Trypsin hydrolyzes peptide chains at the carboxyl side of the amino acids lysine and/or arginine. It is used for numerous biotechnological processes such as protein primary structure analysis, in the breakdown of casein in milk for baby food, and in the development of cell and tissue culture protocols.^{19–22} Immobilization of trypsin significantly reduces autolysis and can be also used to increase trypsin concentration per unit volume by 10–1000-fold. To minimize autodigestion, trypsin is typically used in solution at a 50:1 protein-to-trypsin ratio.^{22,23} Therefore, immobilized trypsin affords an attractive alternative to solution protein digestion.

In the presented work, magnetic beads were prepared by thermal coprecipitation reaction using Fe(III)-incorporated poly(2-hydroxyethyl methacrylate-ethyleneglycol dimethacrylate) (p(HEMA-EGDMA)) beads and Fe(II) ion solution. The magnetic beads were grafted with a hydrophilic fibrous polymer, and the enzyme “trypsin” was covalently immobilized on beads via click chemistry. Hartmuth et al. realized the click chemistry reaction in aqueous medium with Cu(I) as catalyst under very mild conditions.²⁴ The reaction is irreversible, quantitative, and mildly processed. The reaction has been recently used in immobilization of many biological molecules. To the best of our knowledge, the polymer-grafted magnetic

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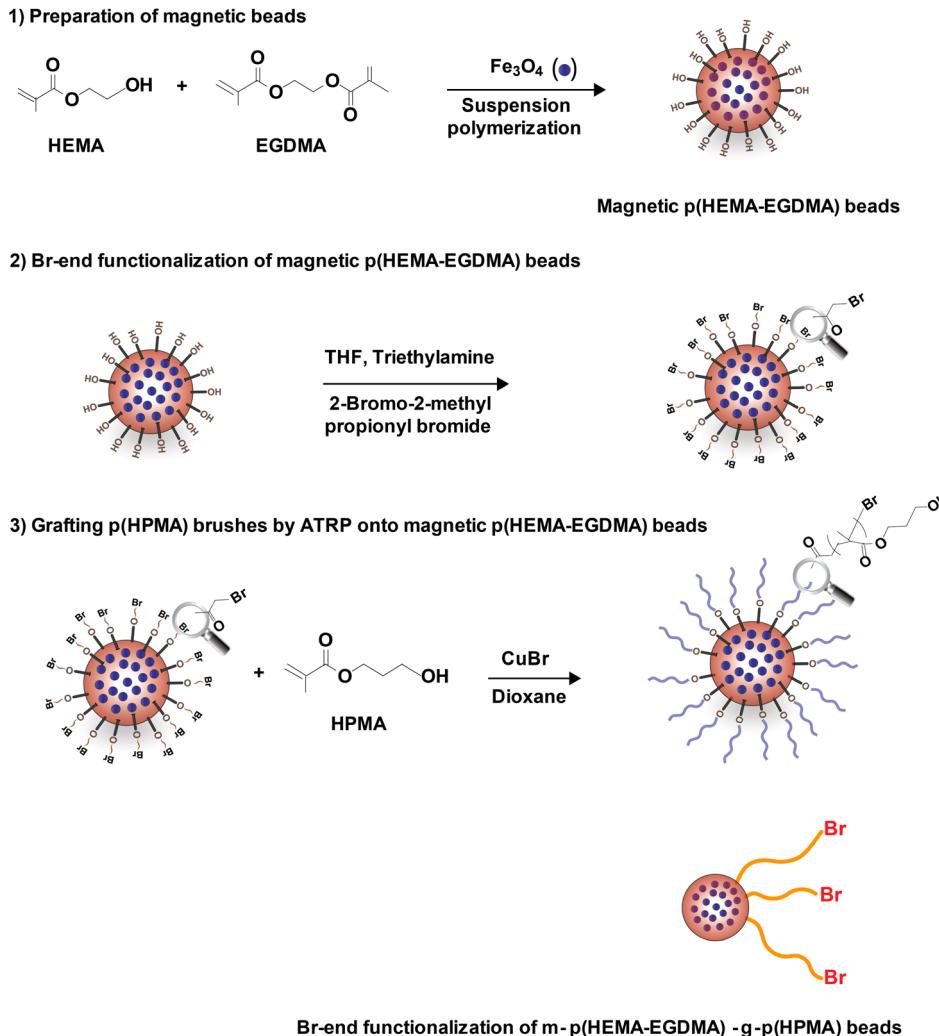


Figure 1. Schematic representation of the preparation of magnetic beads.

beads were first used for immobilization of trypsin via click chemistry. Chemical and physical properties of the trypsin-immobilized magnetic beads were investigated using various characterization tools including surface area, pore size analyzer, FT-IR, SEM, picnometer, and magnetometer. The activity and stability of the immobilized trypsin were investigated under different experimental conditions. Finally, the immobilized trypsin was employed for digestion of native and denaturized model proteins (i.e., cytochrome *c*, lysozyme, and bovine serum albumin (BSA)) in a microbioreactor. To evaluate the digestion performance of the immobilized trypsin, protein digests were analyzed using matrix-assisted laser desorption/ionization mass spectrometer (MALDI-ToF-MS). This method is one of the most important analytical tools used in proteomic studies for peptide mapping.^{23–29} MALDI-ToF-MS results demonstrated that the trypsin-immobilized magnetic beads can be successfully used for efficient protein digestion in proteomic studies and have many important advantages such as reusability, ease of use, and low cost. According to these results, the presented trypsin immobilization strategy could open a new avenue for the application of grafted polymer chains in click chemistry.

MATERIALS AND METHODS

Materials. Trypsin (EC 3.4.21.4; from bovine pancreas type XI; about 7500 U/mg solids), cytochrome *c* (bovine heart),

lysozyme (hen egg white), BSA, 2-hydroxyethyl methacrylate (HEMA), hydroxylpropyl methacrylate (HPMA), ethylene glycol dimethacrylate (EGDMA), and α -cyano-4-hydroxycinnamic acid (α -CHCA) were supplied by Sigma-Aldrich Chem Co. (Germany). Inhibitor from monomers (i.e., HEMA, HPMA, and EGDMA) was removed using aluminum oxide fine coarse powder (Sigma-Aldrich, type CG-20) in a column (diameter = 2.5 cm; height = 20 cm). Bipyridine, water-soluble carbodimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), dimethylformamide (DMF), L-ascorbic acid, sodium azide, Cu(I), CuBr, 2-bromo-2-methylpropionyl bromide, triethylamine, tetrahydrofuran (THF), acetonitrile (ACN), methanol, aqueous ammonia, trifluoroacetic acid (TFA), propionic acid, dimethyl sulfoxide, and α,α -azobis(isobutyronitrile) (AIBN) were supplied from Sigma-Aldrich Chemical Co. All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). All solutions were prepared using ultrapure water.

Preparation of Magnetic Beads. The p(HEMA-EGDMA) beads were prepared via suspension polymerization in the presence of an initiator (i.e., AIBN) as reported previously.¹³ Briefly, the discontinuous phase contained HEMA (10.0 mL), EGDMA (10.0 mL; as cross-linker), and 5.0% polyvinyl alcohol (20.0 mL, as stabilizer) and was mixed together with 0.2 g of AIBN as initiator in 20.0 mL of isopropyl

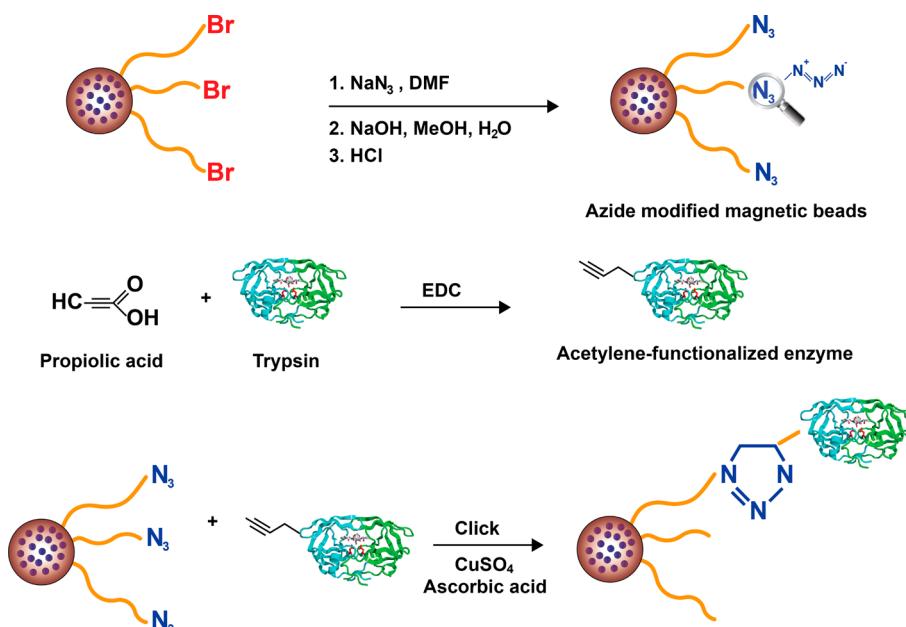


Figure 2. Schematic representation of trypsin immobilization via click chemistry.

alcohol. The aqueous dispersion medium comprised FeCl_3 solution (0.3 M, 400.0 mL), which was used as a precursor for the thermal iron oxide precipitation in the beads. The reaction was maintained at 70 °C for 2.0 h and then at 80 °C for 1.0 h. After the reaction, the beads were collected and washed with distilled water and ethanol.

The magnetization reaction was carried out by conventional coprecipitation reaction of iron oxide in the beads (Figure 1). For the thermal coprecipitation reaction, FeCl_2 (5.0 g) was dissolved in purified water (100.0 mL). It was then transferred into a round-bottom glass flask containing ferric-p(HEMA-EGDMA) beads (15.0 g) in $\text{NH}_3\text{-H}_2\text{O}$ (50.0 mL, 25% w/v). The reaction was carried out under nitrogen atmosphere at two different temperatures (50 and 90 °C each for 3.0 and 2.0 h, respectively) with continuous stirring. Finally, the magnetic beads “mp(HEMA-EGDMA)” were separated using a magnet, washed with ethanol/water (1:1 ratio) for 2.0 h, and then washed with purified water.

Br-End Surface Functionalization of Magnetic mp(HEMA-EGDMA) Beads. The mp(HEMA-EGDMA) beads (about 5.0 g), tetrahydrofuran (50.0 mL), and triethylamine (2.0 mL) were transferred into a flask and stirred magnetically at 100 rpm. Then, 2-bromo-2-methylpropionyl bromide (2.0 mL) was added dropwise to the flask within 30 min. The mixture was incubated at room temperature for 5.0 h. After reaction, the Br-end functionalized beads were removed using a magnet and washed with acetone and purified water. They were then dried under reduced pressure at 35 °C for 1 day before grafting.

Grafting of p(HPMA) Brushes by ATRP onto mp(HEMA-EGDMA)-Br Beads. Graft polymerization of hydroxypropyl methacrylate (HPMA) was achieved through alkyl halide functionalized sites on the surface of the mp(HEMA-EGDMA)-Br beads.⁴ A typical procedure is as follows: p(HEMA-EGDMA)-Br beads (about 5.0 g) were transferred into a glass round-bottom flask (100.0 mL), and monomer HPMA (15.0 mL), CuBr (0.3 g), bipyridine (1.4 g), and dioxane (15.0 mL) were added. The reaction medium was purged with nitrogen for about 10 min and then sealed. The

grafting reaction was carried out at 65 °C for 12.0 h. After the reaction, the reaction content was transferred into acetone (250.0 mL) and stirred magnetically to remove polymerization impurities. The p(HPMA) grafted mp(HEMA-EGDMA)-Br beads were transferred into a solution containing EDTA at about 2.0% (w/v) and stirred continuously to remove copper ions for 48.0 h. The mp(HEMA-EGDMA)-g-p(HPMA)-Br beads were washed sequentially with water (500.0 mL) and ethanol (250.0 mL) and dried in a vacuum oven at room temperature.

Creation of Azide Functionality on mp(HEMA-EGDMA)-g-p(HPMA)-Br Beads. Sodium azide (NaN_3) was reacted with terminal bromine groups of the mp(HEMA-EGDMA)-g-p(HPMA)-Br beads to generate an azide functionality (Figure 2). Briefly, the magnetic beads (about 2.0 g) were transferred into a round-bottom flask containing DMF (20.0 mL), NaN_3 (1.0 g), and distilled water (0.5 mL). The flask was sealed and incubated in the dark at 25 °C while shaking at 150 rpm for 18 h. The azide-functionalized beads were collected with a magnet and washed sequentially with water (100.0 mL), DMF (50.0 mL), and methanol (50.0 mL). The resultant beads were dried for 12.0 h at 50 °C under reduced pressure.

Acetylene Functionalization of Trypsin. Trypsin (100 mg), propionic acid (40 mg), and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; 20 mg) were suspended in 20.0 mL of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5, 10 mmol/L). The resulting mixture was stirred at 4 °C for 8 h for modification of enzyme with acetylene groups (Figure 2). After the reaction, the solution was dialyzed at 4 °C against the same fresh MES buffer solution to remove small molecules for 24.0 h.

Immobilization of Trypsin onto Azide-Functionalized Beads via Click Chemistry. The azide-functionalized beads (2.0 g) were equilibrated in MES buffer (10.0 mL, pH 5.5, 20 mmol/L) at 4 °C for 18.0 h. Copper catalyst solution was freshly prepared using CuSO_4 (10 mmol/L) and ascorbic acid (20 mmol/L) in the same MES buffer solution (10.0 mL). Acetylene-functionalized trypsin in MES buffer solution (20.0 mL) was combined with the above azide-functionalized beads.

The resulting mixture was shaken at 150 rpm at 4 °C for 10.0 h. Finally, the trypsin-immobilized mp(HEMA-EGDMA)-g-p-(HPMA) beads were washed with MES buffer (pH 5.5, 20 mmol/L) and stored at 4 °C until use.

Characterization of Magnetic Beads. The average size and size distribution of the magnetic beads were determined by screen analysis using molecular sieves. The specific surface area of the beads was measured by a surface area apparatus (Quantachrome Nova 2200 E, USA) and calculated using the BET (Brunauer, Emmett, and Teller) method.³⁰ The FTIR spectra of the mp(HEMA-EGDMA), mp(HEMA-EGDMA)-Br, mp(HEMA-EGDMA)-g-p(HPMA), and mp(HEMA-EGDMA)-g-p(HPMA)-enzyme beads were obtained using an FTIR spectrophotometer (Shimadzu, FTIR 8000 series, Japan). Scanning electron micrographs of the dried mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads were obtained using a JEOL JMS 5600 scanning electron microscope (SEM) after coating with gold under reduced pressure. It was operated in the high-vacuum mode with an acceleration voltage of 15 kV. The magnetization curves of the mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads were determined with a vibrating sample magnetometer (VSM, model 155, Digital Measurement System, Inc., Westwood, MA, USA). The weight fraction of iron oxide (Fe_3O_4) in the magnetic beads was determined gravimetrically. The density of magnetic beads was determined by using a stereopycnometer (Quantachrome, SPY 3, USA). The swelling ratio of the beads was determined at 25 °C using a gravimetric method as described previously. After magnetic separation, the amount of trypsin in supernatant solutions was analyzed according to the Bradford method³¹ using a double-beam UV-vis spectrophotometer (PG Instrument Ltd., model T80+, PRC).

Activity Assay of Free and Immobilized Trypsin. Trypsin will hydrolyze ester and amide linkages of several synthetic substrates. The amidase activity of the free and immobilized trypsin was measured by using artificial substrate BA_pNA. In the determination of activity of the free enzyme, the reaction medium consisted of Tris-HCl buffer (pH 7.5, 50 mmol/L, 2.5 mL) containing 10 mM CaCl₂ and 0.1 mL of BA_pNA (0.5 mol/L BA_pNA in DMSO). The reaction mixtures were preincubated in a water bath at 25 °C for 5 min, and the assay was started by the addition of the enzyme solution (0.1 mL) into the assay medium. After 5 min, the reaction was stopped by the addition of 0.3 mL of 30% acetic acid solution. After enzymatic hydrolysis of BA_pNA, *p*-nitroanilide is formed. The amount of *p*-nitroanilide released was monitored via the increase in absorbance at 410 nm. Therefore, the activity of the enzyme can be calculated using the extinction coefficient of *p*-nitroanilide ($\epsilon = 8270 \text{ M}^{-1} \text{ cm}^{-1}$). For the determination of immobilized trypsin activity, 10 mg of trypsin-immobilized beads was introduced to the reaction medium instead of free enzyme solution as described above. The activity of the immobilized trypsin was presented as a percentage of the activity of free enzyme of the same quantity.³²

The effects of pH and temperature on the free and immobilized trypsin preparations were studied in the pH range of 4.0–9.0 and temperature range of 20–60 °C, respectively. The results of dependence on pH and temperature are presented in a normalized form with the highest value of each set being assigned the value of 100% activity. The enzyme activity assay experiments were performed in triplicate. For each set of data, the arithmetic mean values and standard deviations were calculated, and the margin of error for each

data set was determined according to a confidence interval of 95% using the statistical package under Excel for Windows. A value of $P < 0.05$ was considered to be statistically significant.

Determination of the Kinetic Constants. The activity of free and immobilized trypsin was measured at 25 °C and pH 7.5 with various initial concentrations of BA_pNA (from 5 to 50 mmol/L). The relationship between the initial rate (V) of the enzymatic reaction and BA_pNA concentration (S) in phosphate buffer (50 mmol/L) was measured, from which the Michaelis constants K_m and V_{max} of the free and immobilized trypsin preparations were determined. The K_m and V_{max} values for the free and immobilized trypsin preparations were calculated from Lineweaver–Burk plots using the initial rate of the enzymatic reaction.

$$1/v = \{(K_m/V_{max}) \times [1/S] + (1/V_{max})\} \quad (1)$$

[S] is the concentration of substrate, v and V_{max} represent the initial and maximum rates of reactions, respectively, and K_m is the Michaelis constant.

Thermal Stability of Free and Immobilized Trypsin Preparations. The thermal stabilities of free and immobilized trypsin were determined by measuring the residual enzymatic activity of two different temperatures (at 55 and 65 °C) in a phosphate buffer (50 mmol/L, pH 7.5) for 2.0 h. After every 15 min time interval, a sample was removed and assayed for enzymatic activity as described above. The results are given as percent activity.

Operation and Storage Stability of Immobilized Trypsin. The operation stability of the immobilized trypsin in terms of repetitive uses was performed in 20 successive measurements using BA_pNA as substrate on the same day. After enzymatic reaction, the immobilized trypsin was collected by external permanent magnet and washed two times with phosphate buffer (pH 7.5, 50 mmol/L) to remove any residual substrate from the beads. The same trypsin-immobilized magnetic beads were then reintroduced into fresh reaction medium to restart a new run. The remaining activity of the immobilized trypsin was expressed as the percentage of residual activity relative to the initial one.

Storage stability was determined by incubating the immobilized enzyme preparations in phosphate buffer (pH 7.5, 50 mmol/L) at 4 °C for 8 weeks. The remaining enzyme activity was determined with BA_pNA as above. The activities of the free and immobilized trypsin preparations were expressed as a percentage of remaining activity compared to their initial activities.

Digestion of Proteins in Microbioreactor Using Immobilized Trypsin. The enzymatic digestions of cytochrome *c*, lysozyme, and BSA were carried out in a microbioreactor. The microbioreactor was constructed from Pyrex glass (height = 5 cm; inner diameter = 0.2 cm, total volume about 0.16 cm³). About 50 mg of trypsin-immobilized beads was transferred into the microbioreactor. The cytochrome *c*, lysozyme, and BSA (2 mg/mL) were individually dissolved in phosphate buffer (2.0 mL, 50 mmol/L, pH 7.5). The buffer solution containing protein sample was pumped through the bottom inlet part of the microbioreactor by means of a peristaltic pump at 10.0 mL/h. The reactor was operated in dead-end mode at 25 °C. After predetermined time intervals, a sample (50 μL) was removed and analyzed by MALDI-ToF-MS. BSA was reduced with 1,4-dithiothreitol (DTT; 0.1 mL, 10 mM) at 60 °C for 30 min. The solution was allowed to cool to room temperature, and then the reduced cysteine residues

were alkylated with iodoacetamide (0.1 mL, 100 mM). The alkylation reaction was carried out in the dark at room temperature for 30 min. Before trypic digestion of BSA, DTT (20 μ L, 10 mmol/L) was added and digested as described above.

MALDI Mass Spectrometry. MALDI matrix α -cyano-4-hydroxycinnamic acid (α -CHCA) and sinapinic acid (SA) solutions (10 mg mL⁻¹) were prepared in ACN/water/TFA mixture (1:1:0.001, v/v/v). Then, the cytochrome *c*, lysozyme, and BSA digest solutions were mixed with the α -CHCA matrix solution at 1:10 (v/v) ratio, and intact protein solutions were mixed with the SA matrix solution at 1:10 (v/v) ratio by gentle stirring using a vortex. A 1.0 μ L portion of all the final solutions was directly spotted onto the MALDI sample target and dried at room temperature before analysis. MALDI analysis of the samples was carried out in positive ion mode using a Voyager-DE Pro time-of-flight mass spectrometer (Applied Biosystems, USA) with delayed extraction. Ions, formed by a pulsed UV laser beam (N₂ laser, λ = 337 nm) at around 10⁻⁷ Torr, were accelerated with a 23 kV electrical potential. All spectra were the average of 100 shots and internally calibrated.

RESULTS AND DISCUSSION

Characterization of mp(HEMA-EGDMA)-g-p(HPMA) Magnetic Beads. The mp(HEMA-EGDMA) beads were synthesized as reported elsewhere.⁴ After thermal magnetization reaction, the beads were grafted with a hydrophilic p(HPMA) polymer via SI-ATRP. This method was selected for the following reasons: (i) fibrous polymers can be grafted on the magnetic beads in a controlled way; and (ii) after grafting acrylate monomers via ATRP, a secondary bromine end group is available at the end of each fibrous polymer for further modification reactions.^{33–35} For SI-ATRP reaction, first, the hydroxyl groups of the mp(HEMA-EGDMA) polymer were reacted with 2-bromo-2-methylpropionyl bromide to produce bromide-ended beads. The bromine content of the beads was found to be 0.37 mmol Br/g magnetic beads. The grafted polymer chains are also important criteria to increase flexibility of the grafted polymer chains. The reaction rates of functionalized groups with target molecules increase as the distance from the bead core increases. This has been referred to as the “spacer chain effect” and reported in the literature by many authors.^{10,36} The weight gain of grafted polymer on the beads was found to be 380% after 12 h of grafting reaction. The average degree of HPMA unit can be calculated from these data as $[(3.8/144)/(1 + 3.8)] \times 10^3 = 5.498$ mmol of HPMA/g of the (with 3.8 g loaded p(HPMA)/g) magnetic beads and 144 g/mol is the molar mass of HPMA monomer.⁴ If the grafted polymer mass is divided by the initial bromine content (0.37 mmol/g), then the average degree of the polymerization per initiation site would be about 15 units ($5.71/0.227 = 15$ units). The bromide groups of the p(HPMA) grafted beads were reacted with sodium azide to obtain azidated beads, and the functionalized beads were used for immobilization of trypsin via click chemistry.

The surface morphologies of the mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads were investigated by SEM (parts A and B, respectively, of Figure 3). As seen in this figure, the morphology of the polymer-grafted beads is distinctly different as compared to the nongrafted counterpart. The fibrous polymer grafted magnetic beads can be a suitable support due to their intrinsically high specific surface area with azide functional groups. Thus, the p(HPMA) grafted and azide-

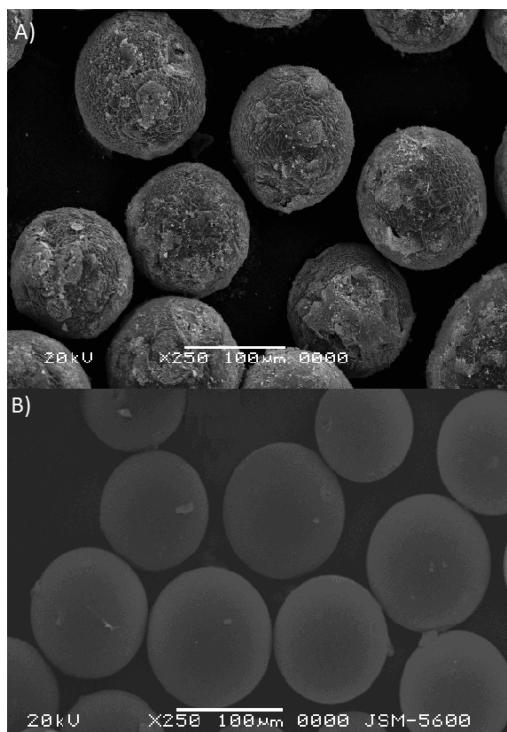


Figure 3. SEM micrographs: (A) mp(HEMA-EGDMA) beads; (B) mp(HEMA-EGDMA)-g-p(HPMA).

modified polymer chains can provide a high quantity of binding sites for immobilization of various biological molecules. The SEM photograph indicates that magnetic p(HEMA-EGDMA) beads have a highly porous surface predominantly including macropores (Figure 3A). As seen from Figure 3B, the pores of the beads were filled by the grafted p(HPMA) polymer, and this was also indicative of successful SI-ATRP grafting. Some properties of the magnetic mp(HEMA-EGDMA)-g-p(HPMA) beads are summarized in Table 1.

Table 1. Some Properties of the mp(HEMA-EGDMA)-g-p(HPMA) Magnetic Beads

bead size	75–150 μ m
bromine groups content	0.37 mmol/g
average p(HPMA) units	15
surface area bare beads	13.7 m ² /g
swelling ratio	54%
density	1.17 g/cm ³
weight fraction of polymer	87.8%
weight fraction of Fe ₃ O ₄	12.2%

FTIR analysis provides information regarding the changes on surface functional groups of the beads. Figure 4 shows FTIR spectra of the mp(HEMA-EGDMA), mp(HEMA-EGDMA)-g-p(HPMA), and mp(HEMA-EGDMA)-g-p(HPMA)-trypsin beads. As seen in Figure 4A, the FTIR spectra of p(HEMA-EGDMA) have the characteristic stretching vibration band of hydrogen-bonded alcohol of HEMA at around 3429 cm⁻¹. Among the characteristic vibrations of both HEMA and EGDMA are the methylene vibration at 2933 cm⁻¹. The vibration at 1719 cm⁻¹ represents the ester configuration of both HEMA and EGDMA. In addition, the characteristic band of the Fe=O at 568 cm⁻¹ is also observed. On the other hand,

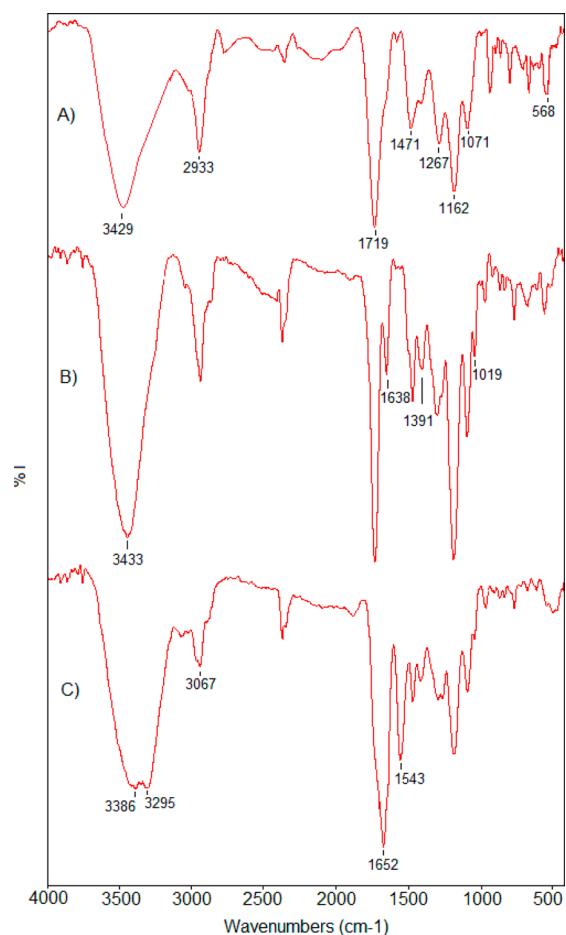


Figure 4. FTIR spectra: (A) mp(HEMA-EGDMA) beads; (B) mp(HEMA-EGDMA)-g-p(HPMA); (C) mp(HEMA-EGDMA)-g-p(HPMA)-trypsin.

the relative intensity of the hydroxyl band at 3433 cm^{-1} compared to mp(HEMA-EGDMA) increased in the FTIR spectra of p(HPMA) grafted beads (Figure 4B). The sharp band at 3295 cm^{-1} in the FTIR spectrum of the mp(HEMA-EGDMA)-g-p(HPMA) beads after conjugation of trypsin clearly indicated the presence of a triazole ring on the trypsin-immobilized magnetic beads (Figure 4C). Additionally, the FTIR spectra of the trypsin immobilized mp(HEMA-EGDMA)-g-p(HPMA) beads have the characteristic N–H amine stretching bands between 3500 and 3400 cm^{-1} (the overlapped bands with –OH groups became broader and expanded after immobilization of enzyme via click reaction). The most important new absorption band at 1543 cm^{-1} representing N–H bending is due to the amino groups formed during click reaction between the enzyme and the functionalized support. These results indicated that click chemistry was successfully used for the covalent immobilization of trypsin onto the mp(HEMA-EGDMA)-g-p(HPMA) beads.

The presence of iron oxide crystals within the mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads was also determined by VSM. The plots of magnetization versus magnetic field curve for the mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads at room temperature are presented in Figure 5. The specific saturation magnetization (σ_s) was found to be 24.7 and 13.9 emu/g for mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads, respectively. The low saturation magnetization value of mp-

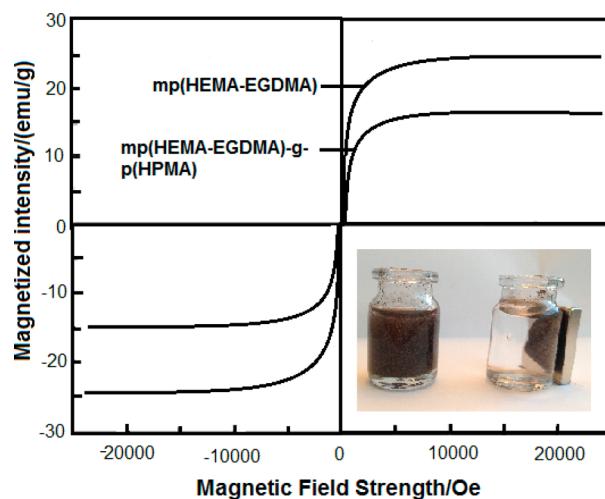


Figure 5. Magnetization versus magnetic field for mp(HEMA-EGDMA) and mp(HEMA-EGDMA)-g-p(HPMA) beads at room temperature. Additionally, separation of dispersed magnetic beads from the medium with an external magnet is also presented.

(HEMA-EGDMA)-g-p(HPMA) beads compared to pristine magnetic beads can be due to the presence of extra polymer shells. The diamagnetic contribution of the extra polymer shells surrounding the grafted magnetite beads weakened the magnetic moment. However, both of them showed superparamagnetic behaviors, indicating that grafting of the magnetic beads exhibited no remaining effect from the hysteresis loops at the applied magnetic field. Additionally, separation of dispersed magnetic beads from the solution in the presence of an external magnetic field is also presented in Figure 5. Thus, an enzymatic reaction can be stopped at the desired time by easily separating enzyme containing magnetic responsive beads with a conventional permanent magnet. After removal of the magnetic force, the magnetic responsive composite beads can easily be resuspended in the reaction medium by simple shaking.³⁷

Effect of Copper Ions on Activity of Free Trypsin. The free trypsin preparation (1.0 mg/mL) was incubated in Tris–HCl buffer solution ($\text{pH } 7.5$, 50 mmol/L , 5.0 mL) in the presence and absence of copper ions at $4\text{ }^\circ\text{C}$ for 18 h , and the remaining activity of the trypsin preparation was determined as described above. It was observed that the copper ion had a negative effect on the trypsin activity in the presence of Cu(II) ions (i.e., CuSO_4 , 10 mmol/L), causing a reduction of trypsin activity of about 8% compared to the activity of trypsin incubated at $4\text{ }^\circ\text{C}$ in the absence of Cu(II) ions for 18 h .

Trypsin Immobilization Studies. Trypsin is commonly used to digest proteins at arginine and/or lysine residues, and this enzymatic digestion is very important for mass spectrometric sequencing because it generates peptides that have molecular weights within the mass range of the MS for amino acid sequence determination.^{18–20} The immobilization of trypsin on the beads was carried out with two-step click chemistry reaction. In the first step, NaN_3 was reacted with terminal bromo groups of the magnetic beads to modify the azide functionality. In the second step, an alkyne-functionalized trypsin was obtained in MES buffer solution. The formation of an ester bond between trypsin and alkyne carrying agent (i.e., propionic acid) was realized using carbodiimide as coupling agent. Thus, the immobilization of trypsin via click reaction was realized by reacting the alkyne-functionalized enzyme with the azide-modified magnetic beads. In this reaction, triazole

formation is irreversible, and a 1,3-dipolar cycloaddition reaction proceeds in the presence of Cu(II) ion as catalyst. The amount of immobilized trypsin on the mp(HEMA-EGDMA)-g-p(HPMA) beads via click chemistry was found to be about 18.3 mg/g, and the retained activity yield of the immobilized trypsin was found to be 75.7%. To the best of our knowledge, this is the first study to make use of click chemistry for the covalent immobilization of trypsin.

A variety of methods have been developed for trypsin immobilization; for example, trypsin was covalently immobilized on the hybrid magnetic nanoparticles with hairy non-cross-linked polymer chains for digestion of protein. Silica-coated magnetic nanoparticles were grafted with poly(glycidyl methacrylate) (p(GMA)) via the SI-ATRP method. The epoxy groups of the grafted p(GMA) chains were converted to an aldehyde group for trypsin immobilization. The trypsin immobilization capacity of the support was found to be 247 $\mu\text{g}/\text{mg}$, which was higher than that of the presented trypsin immobilization method, 18.3 $\mu\text{g}/\text{mg}$ (or 18.3 mg/g). This low immobilization capacity of the presented method can be due to specific binding reaction between activated enzyme and activated support via click chemistry.³⁸ Chen et al. successfully immobilized trypsin via click reaction to the hybrid organic-inorganic monolithic capillary column with thioene functionality. The amount of immobilized trypsin was about 4.62 mg/cm to the capillary microreactor and retained 87.5% of its initial activity.³⁹ Schlossbauer et al. studied the covalent immobilization of trypsin on the large-pore SBA-15 support via click chemistry approach. The retained activity of the immobilized trypsin was about 20% compared to the same quantity of free enzyme.⁴⁰ In the presented study, the immobilized trypsin retained 75.7% of its initial activity. Thus, the presented immobilization method is comparable with the related literature.

Effect of pH and Temperature on Free and Immobilized Trypsin. The effect of pH on the activity of free and immobilized trypsin in BA_nNA hydrolysis was carried out in the pH range 4.0–9.0 (Figure 6). As seen in this figure, optimal conversion was observed at pH 7.5 for the free enzyme, whereas the optimum pH value was slightly shifted to the alkaline region (i.e., pH 8.0) upon immobilization. The immobilized trypsin has a different pH activity profile compared to free enzyme and was broadened in both alkaline

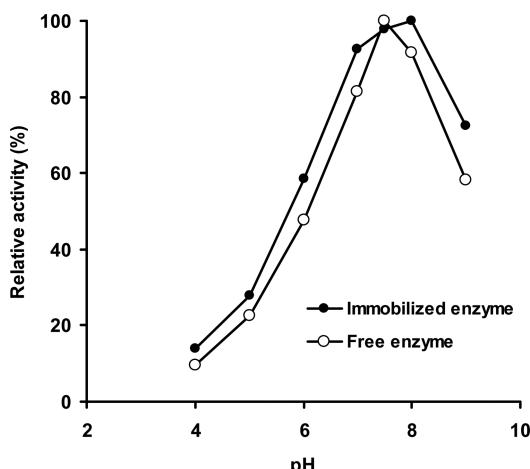


Figure 6. Effect of pH on catalytic activity of the free and immobilized trypsin preparations.

and acidic regions. Additionally, compared to the free enzyme, immobilized trypsin retained >90% activity over a wider pH range of 7.0–8.5. This result could be attributed to the basic character of the triazole ring around the microenvironment of the immobilized enzyme. The triazole ring might show a buffering effect, and the immobilized trypsin may be less affected by the acidity of the reaction medium. In addition, the hydrophilic polymer chains could also provide a comfortable microenvironment to stay in the active conformation for the immobilized trypsin. Under this condition, the active site could become more exposed to medium than the free form for binding of its substrate. Other researchers have reported similar observations upon immobilization of trypsin and other enzymes.^{6,41}

The temperature dependence of the activity of the free and immobilized trypsin was studied in the temperature range of 20–60 °C (Figure 7). Optimum temperature was found at

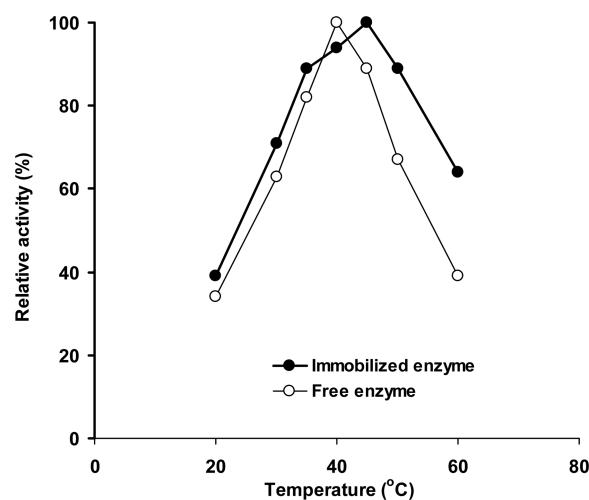


Figure 7. Effect of temperature on catalytic activity of the free and immobilized trypsin preparations.

about 40 °C for free trypsin and at 45 °C for the immobilized trypsin. A plateau region is seen for immobilized enzyme at temperatures between 45 and 55 °C that was not apparent for the free enzyme. Additionally, immobilized trypsin yielded higher activity over a wider range of temperatures than did the free trypsin. The increase in optimum temperature for the immobilized enzyme could be due to the change in physical and chemical properties during the modification process. The applied immobilization protocol and covalent binding might also reduce the flexibility of the trypsin molecule and result in an increase in the activation energy of the enzyme to bind its substrate.

Kinetic Parameters of Free and Immobilized Trypsin. Kinetic parameters V_{\max} and K_m for the free and immobilized trypsin were calculated from Lineweaver–Burk plots at constant temperature and pH while the artificial substrate (i.e., BA_nNA) concentration was varied. The V_{\max} and K_m values were found to be 7684 and 5865 U/mg and 8.6 and 16.7 mmol/L for free and immobilized enzyme, respectively. The Michaelis constant K_m might be affected by the increased diffusion limitation, which results in a lower possibility of forming an enzyme–substrate complex.⁴² The K_m value for immobilized trypsin was 1.91-fold higher compared to that of the free trypsin, possibly due to the diffusional limitation imposed on the flow of substrate and product molecules from

the grafted polymer layer of the magnetic beads. The grafted polymer and the immobilized enzyme layers could create a sticky web structure and may prevent the diffusion of the substrate and/or products between these layers. V_{\max} defines the highest possible reaction velocity when all of the enzyme molecules are saturated with substrate. The efficiency factor (η) can be found from the V_{\max} value of the immobilized trypsin over that of its free enzyme. The efficiency factor for the immobilized trypsin was calculated as 0.762.

Thermal Stability of the Free and Immobilized Trypsin. Thermal stability experiments were studied at two different temperatures with the free and immobilized trypsin. The results are shown in Figure 8. The heat inactivation rate of

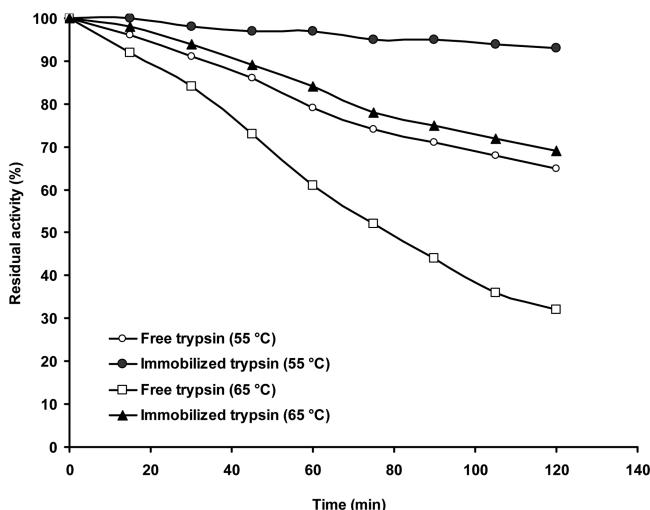


Figure 8. Thermal stability of the free and immobilized trypsin at 55 and 65 °C.

the immobilized trypsin was smaller than that of the free enzyme. At 55 °C, the free enzyme retained 65% of its initial activity during a 120 min incubation period, whereas the immobilized trypsin on the magnetic beads preserved about 93% its initial activity after incubation for the same period. At 65 °C the free and immobilized enzymes retained their activity to levels of about 22 and 69%, respectively. It should be noted that each trypsin molecule surface has 15 lysine and 2 arginine residues. Hence, the trypsin molecule should be immobilized on the support with multipoint covalent linkage. The improvement in the stability of the immobilized enzyme can be due to the restriction in the conformational flexibility of the immobilized trypsin molecule, as a result of multipoint covalent binding between the enzyme and the support.^{32,43} Additionally, the improved stability of the immobilized trypsin could be also due to the reduction in the self-autolysis rate of the trypsin compared with the freely suspended trypsin in solution. The extent of autolysis in the inactivation of trypsin can be difficult to estimate in the freely suspended enzyme. Increased thermal stability of immobilized trypsin preparations has been reported in the literature.^{43–46}

Repeated Use of Immobilized Trypsin. One of the problems in continuous enzymatic reaction is the operational stability of the immobilized enzyme. The operational stability of the immobilized trypsin was studied in batch operation mode for the hydrolysis of the artificial substrate BA_nNA at 30 °C for 20 min. The immobilized trypsin was reused 20 times in one day. After each reaction period, the same enzyme-immobilized

magnetic beads were washed with phosphate buffer (50 mmol/L, pH 7.5) to remove any residual reactant within the enzyme-support system. The initial activity was considered as 100% for the immobilized enzyme. The relative activity of immobilized trypsin decreased with the increase of the reuse number. As shown in Figure 9, the immobilized enzyme retained >84% of its initial activity after 20 consecutive repeated uses. This activity loss might be caused by deactivation of the enzyme upon use.^{32,47,48}

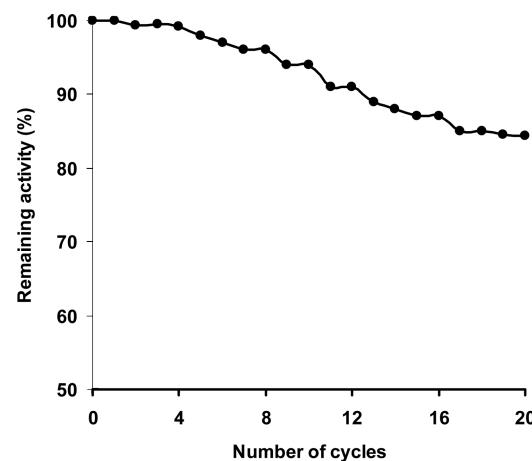


Figure 9. Operational stability of the immobilized trypsin in batch mode.

Storage Stability of Free and Immobilized Trypsin.

The storage stabilities of the free and immobilized enzymes were determined by storage in phosphate buffer (50 mmol/L, pH 7.5) at 4 °C, and the activity measurements were carried out for a period of 8 weeks. The activity of the immobilized trypsin decreased more slowly compared to that of the free enzyme. It lost all of its activity within 3 weeks, whereas the immobilized enzyme lost about 28% of its activity during 8 weeks of storage (Figure 10). The loss of activity for the free enzyme can be due to the autodigestion of the enzyme in storage medium, and immobilization significantly prevented this phenomenon. These results are also in agreement with the literature.^{18,42,44–47}

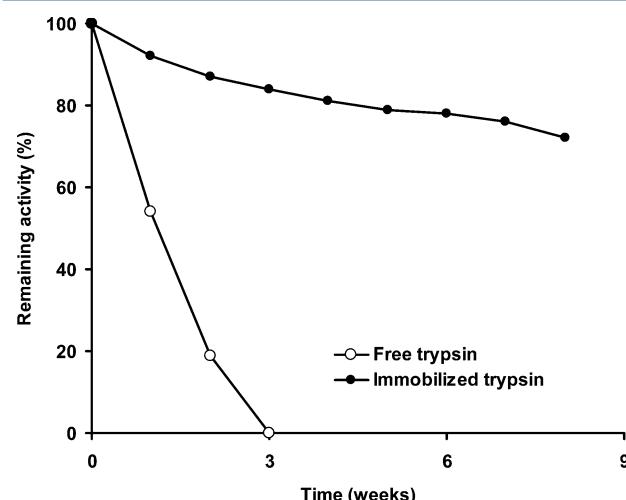


Figure 10. Storage stability of free and immobilized trypsin at 4 °C.

Protein Digestion Efficiency of Immobilized Trypsin.

The usability and efficiency of the immobilized trypsin for protein digestion were tested with three different proteins (i.e., denatured BSA, lysozyme, and cytochrome *c* with molecular weights of 67.0, 14.3, and 12.0 kDa, respectively) in a microbioreactor by dead-end operation mode.³² Enzymatic digestion products of each protein were determined with the MALDI-ToF MS system (Figure 11). BSA was selected as a

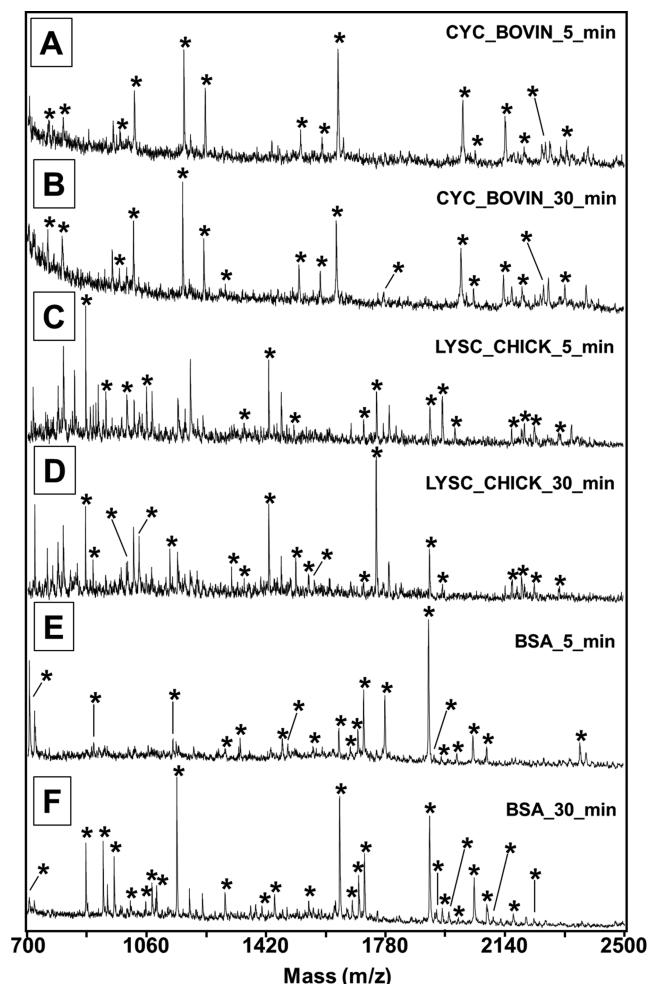


Figure 11. Positive ion and linear mode MALDI-ToF-MS spectra of the digested solution obtained with trypsin immobilized core–shell magnetic beads: (A) cytochrome *c* digestion in 5 min; (B) cytochrome *c* digestion in 30 min; (C) lysozyme digestion in 5 min; (D) lysozyme digestion in 30 min; (E) bovine serum albumin digestion in 5 min; (F) bovine serum albumin digestion in 30 min. * represents the specific enzyme digested peptide fragments.

large MW and acidic protein. The latter two proteins were selected as small MW and basic proteins. As observed in this figure, a large number of tryptic peptides from those three proteins were separated and detected. This result shows that the presented enzyme microbioreactor can be used for online digestion of proteins in analysis and in characterization. As a large globular protein, BSA usually has low sequence coverage in tryptic digestion for the complex structure. As reported earlier, the hydrolysis of BSA is difficult using the usual protocol.²⁷ For this reason, BSA was denatured for efficient tryptic digestion. Additionally, the trypsin-immobilized magnetic beads were tested for enzyme leakage to determine

nonspecific immobilization of trypsin molecules onto magnetic beads in terms of noncovalent interactions between trypsin and fibrous polymer grafted magnetic beads. Therefore, MALDI-ToF-MS analysis was carried out in positive ion mode using sinapinic acid matrix for the detection of proteins. In this spectrum the trypsin signals at around 23 kDa representing enzyme release from the magnetic beads could not be observed (data not shown). This result showed that the covalent immobilization of trypsin via click reaction to the magnetic beads was successfully performed, and there were no nonspecifically and/or fortuitously linked enzymes on the magnetic beads. However, digestion time plays a key role in the proteomics applications and varies between 2 and 24 h in conventional applications. Long digestion periods can lead to the formation of autocatalysis products of trypsin in high amounts. For this reason, short digestion periods are preferred during the study of too many samples in proteomics applications. In this study, digestion is carried out within 5 min for cytochrome *c* and also lysozyme (Figure 11A,C). In the case of BSA, a 5 min digestion time is also acceptable for denatured protein (Figure 11E). However, the best results can be obtained by 30 min of digestion time (Figure 11F), which is also a sufficiently short digestion period for the high molecular weight proteins in proteomics applications.

CONCLUSION

The presented azide–alkyne click reaction approach is a promising method for highly selective immobilization of enzyme under mild conditions. In addition, the biocompatibility of the magnetic beads could be improved by coating with a hydrophilic and biocompatible fibrous polymer so that denaturation and autolysis of the immobilized trypsin may be minimized. The microbioreactor was successfully applied to digestion of three different model proteins. The immobilized trypsin within the microbioreactor coupled with MALDI-ToF MS is a promising strategy for efficient protein digestion and peptide mapping. The presented magnetic beads with azide functionality can also be used as supports for immobilization of various biological molecules such as antibody/antigen and other enzymes. Finally, the fibrous polymer grafted magnetic beads are attracting interest for use in many bioanalytical applications.

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Notes

The authors declare no competing financial interest.

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