New Monodisperse Magnetic Polymer Microspheres Biofunctionalized for Enzyme Catalysis and Bioaffinity Separations

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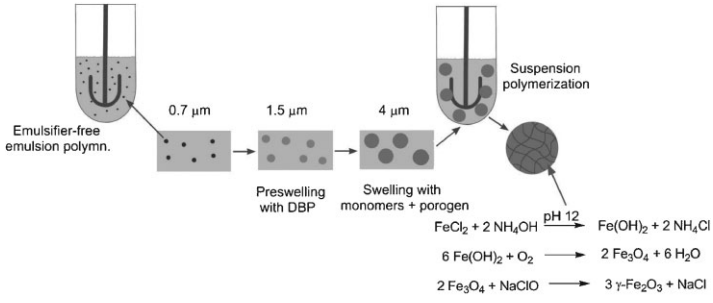
Magnetic macroporous PGMA and PHEMA microspheres containing carboxyl groups are synthesized by multi-step swelling and polymerization followed by precipitation of iron oxide inside the pores. The microspheres are characterized by SEM, IR spectroscopy, AAS, and zeta-potential measurements. Their functional groups enable bioactive ligands of various sizes and chemical structures to couple covalently. The applicability of these monodisperse magnetic microspheres in biospecific catalysis and bioaffinity separation is confirmed by coupling with the enzyme trypsin and huIgG. Trypsin-modified magnetic PGMA-COOH and PHEMA-COOH microspheres are investigated in terms of their enzyme activity, operational and storage stability. The presence of IgG molecules on microspheres is confirmed.



1. Introduction

Interest in superparamagnetic microspheres has been rapidly increasing recently due to their potential in biotechnology and biomedicine. After biofunctionalization, they find practical uses in immunoprecipitations, gentle but highly efficient isolation and purification of biomolecules[1] (proteins, enzymes, antibodies, and nucleic acids), biospecific catalysis,[2] immunomagnetic cell separation,[3] diagnosis and prognosis of malignant diseases,[4] etc. Their main advantages are easy manipulation, high capacity and simple separation from complex heterogeneous biological mixtures including blood, urine, foodstuffs, or tissues. Conventional mechanical methods, such as filtration or centrifugation or multi-step column separation techniques, including size-exclusion chromatography, fractional precipitation, or ion exchange chromatography, can thus be avoided. Moreover, magnetic microsphere-based bioaffinity separation methods enable the effective isolation of viable cells and labile molecules due to the lower mechanical stress than the aforementioned methods.[5]

Several methods are commonly used for the preparation of magnetic polymer microspheres. Encapsulation of magnetic cores with a polymer results in polydisperse particles with irregular shapes,[6] while miniemulsion polymerization in the presence of iron oxide[7] produces

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particles <500 nm, emulsion polymerization[8,9] particles <1 μm, dispersion polymerization[10] around 1 μm, and suspension polymerization provides polydisperse particles measuring hundreds of micrometers.[11] An advantage of the multi-step swelling and polymerization technique pioneered by Ugelstad et al.[12] and elaborated by others[13,14] is that it produces strictly monodisperse magnetic particles larger than 1 μm, which are difficult to produce by other techniques. This technique was therefore for the first time adapted in this report on earlier elaborated by us suspension polymerization of glycidyl methacrylate (GMA) and 2-hydroxyethyl methacrylate (HEMA). Another novelty consists in utilization of a new porogen (cyclohexyl acetate) and new stabilizers [2-hydroxyethylcellulose and (hydroxypropyl)methylcellulose] for preparation of macroporous poly(GMA) or poly(HEMA) (PGMA and PHEMA, respectively) microspheres and also in formation of magnetic iron oxide in the pores by oxidation of $Fe(OH)_2$ obtained by precipitation of neat $FeCl_2$ with ammonia.

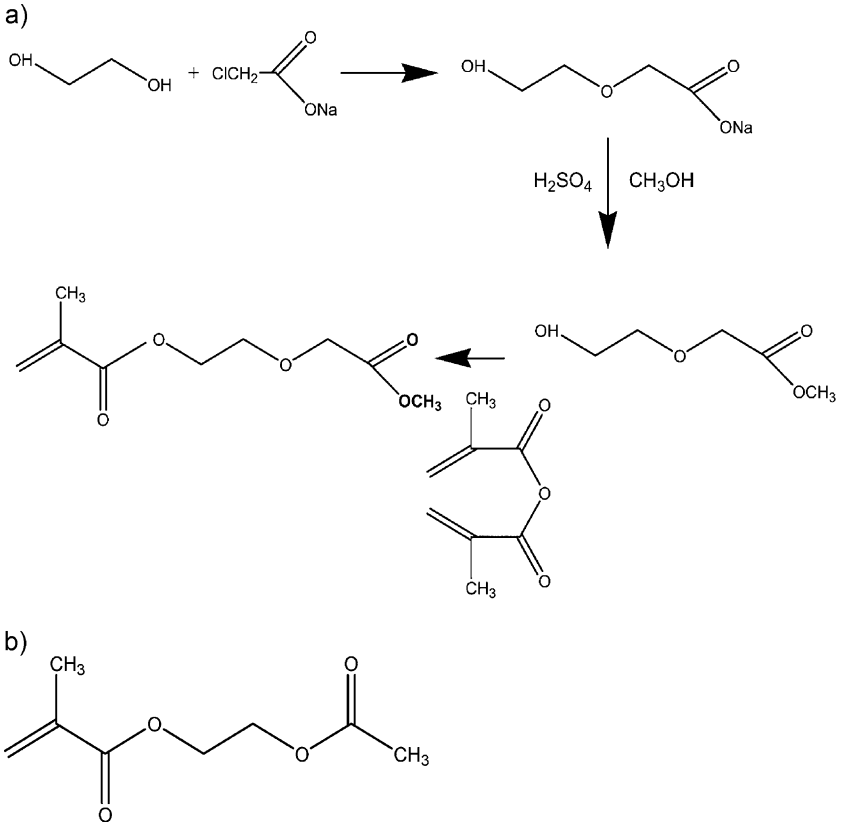
Despite the wide availability of commercial magnetic carriers, they do not often fulfill all the criteria needed for unique bioanalytical research. The main requirements laid on magnetic microspheres include superparamagnetic behavior, proper size, monodispersity, applicability, and stability in various media (aqueous, organic, and mixed) for a given usage.[15–17] The appropriate type of functional groups on the surface enables the covalent attachment of various biomolecules. The density of functional groups is then one of the key parameters of the microspheres. Even though the number of various applications of magnetic microspheres is continually growing, there are still many areas where the use of newly developed magnetic microspheres with suitable features could be beneficial, e.g., micrototal analysis systems.[18,19] Microspheres need to meet particularly specific requirements in such developing fields as microfluidics. Combining microfluidic devices with magnetic microspheres is creating new possibilities in innovative bioapplications.[19,20] This is the reason why attention has been paid to the development of new types of microspheres.

In order to be able to routinely utilize magnetic microspheres, it is vital to deeply characterize them, verify their stability in various media during the activation process as well as during regular usage and confirm the ability to covalently attach suitable bioactive ligands and subsequently isolate the analyte of interest from the complex mixture in the required purity. Due to the increased stability of immobilized ligands, covalent bonds are preferred for a wide range of applications. Hence, bioactive ligands exemplified by the proteolytic enzyme trypsin and human immunoglobulin G (huIgG) were immobilized on magnetic PGMA and PHEMA microspheres containing carboxyl groups (further denoted as PGMA-COOH and PHEMA-COOH microspheres). Trypsin was selected for the simple quantification of its proteolytic activity. Human IgG served to simulate a biologically active high-molecular-weight biocompound that was immobilized on the magnetic microspheres to form an immunosorbent. The amount of IgG immobilized on the microspheres was quantified by standard bioanalytical methods.

2. Experimental Section

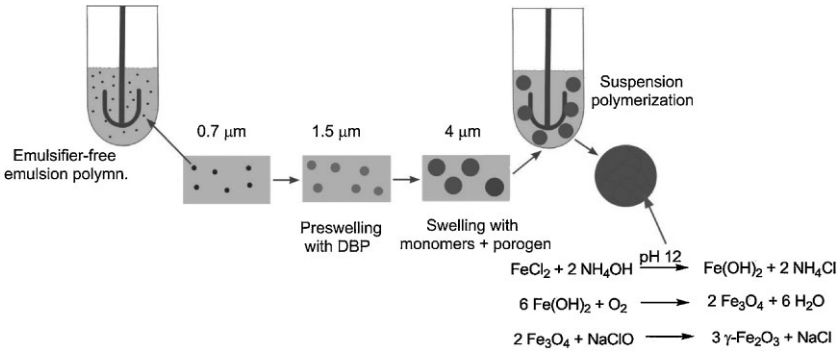
2.1. Materials

Monomers such as styrene (Kaučuk Kralupy, Czech Republic), HEMA (Röhm, Darmstadt, Germany), GMA (Fluka, Buchs, Switzerland), and ethylene dimethacrylate (EDMA; Ugilor S. A., France) were vacuum distilled. 2-[(Methoxycarbonyl)methoxy]ethyl



Scheme 1. Synthesis of (a) MCMEMA and (b) HEMA-Ac.

methacrylate (MCMEMA) was prepared from ethylene glycol (300 mL) and chloroacetic acid (94.5 g) in the presence of NaOH (80 g), producing sodium hydroxyethoxyacetate, which was then transformed (in the presence of H_2SO_4 and methanol) to the methyl ester of hydroxyethoxyacetic acid and finally to MCMEMA using methacrylic anhydride (Scheme 1a). 2-(Methacryloyl)oxyethyl acetate (HEMA-Ac; Scheme 1b) was obtained from HEMA and acetic anhydride. Cyclohexyl acetate was obtained from cyclohexanol and acetic anhydride. Trypsin from bovine pancreas (EC 3.4.22.2), bovine serum albumin (BSA), IgG from human serum, benzamidine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-α-benzoyl-D,L-arginine-4-nitroanilide (BApNA), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Pierce® BCA Protein Assay Kit was produced by ThermoScientific (Rockford, IL, USA). The sodium salt of *N*-hydroxysulfosuccinimide (sulfo-NHS), $FeCl_2 \cdot 4H_2O$, 2-hydroxyethyl cellulose, sodium dodecylsulfate (SDS), benzoyl peroxide (BPO), and Methocel 90 HG [(hydroxypropyl)methyl cellulose] were obtained from Fluka, sodium persulfate was from Lachema (Brno, Czech Republic). Sodium azide was produced by Chemapol (London, United Kingdom). The remaining chemicals were supplied by Sigma-Aldrich, Lachema, or Penta Chemicals (Chrudim, Czech Republic) and were of analytical reagent grade. Ultrapure Q-water ultrafiltered with a Milli-Q Gradient A10 system (Millipore, Molsheim, France) was used for preparing solutions.



Scheme 2. Preparation of monodisperse magnetic macroporous polymer microspheres by multi-step swelling and polymerization method and precipitation of iron oxide inside their pores.

2.2. Synthesis of Monodisperse Polystyrene (PS) Seeds

PS seeds were obtained by the emulsifier-free emulsion polymerization of styrene in a 150-mL reaction vessel equipped with an anchor-type stirrer. In brief, sodium persulfate (44 mg) and sodium carbonate (39 mg) were dissolved in water (90 mL) to form the aqueous phase. The monomer phase (10 g styrene) was added, the mixture stirred (300 rpm), and the temperature increased to 80 °C. Polymerization proceeded for 20 h under a nitrogen atmosphere. The resulting latex was separated by centrifugation (4000 rpm) and thoroughly washed with a 0.25% aqueous solution of SDS.

2.3. Synthesis of Monodisperse Macroporous PGMA-COOH and PHEMA-COOH Microspheres

Macroporous PGMA-COOH and PHEMA-COOH microspheres were synthesized by the modified multi-step swelling and polymerization method originally developed by Ugelstad et al.[12] (Scheme 2). First, PS latex (0.3 g) was dispersed in 0.25% SDS solution (1 mL) and the mixture sonicated (4710 Series Ultrasonic homogenizer; Cole-Parmer, Chicago, IL, USA) at 15 °C for 3 min. Second, the latex was mixed with an emulsion of dibutyl phthalate (DBP; 0.4 g) in 0.25% SDS (1.2 mL) and 2% $NaHCO_3$ solutions (0.05 mL) under sonication at 15 °C for 4 min. PS latex was swollen with DBP for 4 d with mild stirring (30 rpm); swelling was repeated once more with the same amount of DBP and then four times with double the amount of DBP. The resulting PS latex contained 4.3 g DBP in 17.5 mL of dispersion.

Third, DBP-swollen PS particles were swollen with the monomers, porogen, and initiator in a 30-mL reaction vessel. Briefly, a DBP-swollen PS dispersion (2 mL) was swelled with an emulsion of a solution of BPO (30 mg), GMA (1.5 g), MCMEMA (0.3 g), and EDMA (1.2 g) in 0.1% SDS solution (7.5 mL) for 16 h with gentle stirring (30 rpm). A mixture of cyclohexyl acetate (4 g) in 0.1% SDS solution (10 mL) was then treated with ultrasound at 22 °C for 3 min to form an emulsion and transferred to the above monomer-swollen PS dispersion; swelling proceeded for 3 h under stirring (300 rpm). Alternatively, HEMA-Ac replaced GMA in the swelling solution when PHEMA-COOH microspheres were being prepared.

In the fourth step, a 2 wt% solution of 2-hydroxyethyl cellulose (2 mL), 2 wt% solution of Methocel 90 HG (2 mL), and a solution of citric acid (30 mg) in water (0.5 mL) were mixed with the above monomer-swollen PS latex and the polymerization proceeded at 70 °C for 16 h at stirring rate of 600 rpm under a carbon dioxide atmosphere. The resulting microspheres were removed by filtering, washed with 0.05 wt% Tween 20 solution, ethanol, toluene, and ethanol (five times each), and finally transferred to water. In order to change the methyl ester of MCMEMA to a carboxyl group, the PGMA microspheres were separated by centrifugation and hydrolyzed in 0.2 M H_2SO_4 (50 mL) at 22 °C for 60 h. The microspheres were then washed five times with water with ultrasonic treatment. In order to yield PHEMA microspheres, the acetate of poly[2-(methacryloyl)oxyethyl acetate] was hydrolyzed in 0.5 M NaOH solution (30 mL) in the presence of Tween 20 at 60 °C for 16 h with stirring (300 rpm); the microspheres were purified by washing four times with water and twice with 1,4-dioxane.

2.4. Precipitation of Iron Oxide Inside the Macroporous Microspheres

Macroporous PGMA-COOH or PHEMA-COOH microspheres (1 g) were dispersed in a solution of $FeCl_2 \cdot 4H_2O$ (2 g) in water (10 mL) for 2 min with ultrasonic treatment, removed by filtering, washed again with $FeCl_2$ solution, and left to dry at 40 °C for 30 min. They were then transferred to a 0.5 M NH_4OH solution (20 mL), the mixture stirred (100 rpm) in air at 22 °C for 3 h, the particles separated using a magnet, washed several times with water (100 mL), and mixed with 5 wt% sodium hypochlorite solution

(2 mL) with stirring (100 rpm) for 10 min. Finally, the microspheres were washed with water, ethanol, and water. Before trypsin immobilization, the microspheres were stored in distilled water with 0.1% NaN_3.

2.5. Characterization of Magnetic Microspheres

The microsphere morphology, size, and size distribution were analyzed by scanning electron microscopy (SEM; JEOL JSM 6400, Tokyo, Japan). The number-average diameter (D_n), weight-average diameter (D_w), and uniformity (polydispersity index PDI $= D_w/D_n$) were calculated using Atlas software (Tescan Digital Microscopy Imaging, Brno, Czech Republic) by counting at least 500 individual particles from SEM microphotographs. The D_n and D_w can be expressed as follows:

$$D_n = \frac{\sum n_i D_i}{\sum n_i}$$

$$D_w = \frac{\sum n_i D_i^4}{\sum n_i D_i^3}$$

The microspheres were examined with a Paragon 1000 PC FTIR spectrometer (Perkin-Elmer) with a Specac MKII Golden Gate Single Reflection ATR System with a diamond crystal and a ray angle of incidence of 45°. The iron content was analyzed by atomic absorption spectrometry (AAS Perkin-Elmer 3110) of an extract from a sample obtained by treatment with 70% perchloric and 65% nitric acid at 100 °C for 30 min. A 799 GPT Titrino titrator (Metrohm) was used to evaluate the carboxyl group content of the microspheres by titrating with 0.1 M NaOH.

The electrostatic stability of the microspheres (46 $\mu g \cdot mL^{-1}$ of 0.01–0.1 M phosphate buffer; pH = 7.3) was investigated by zeta-potential measurements using a ZetaPALS apparatus (Brookhaven Instruments; New York, USA).

2.6. Immobilization of Trypsin on Magnetic PGMA-COOH and PHEMA-COOH Microspheres

Three immobilization approaches were investigated for the covalent attachment of trypsin to magnetic microspheres: a one-step procedure using the zero-length crosslinker EDC and sulfo-NHS and a one- or two-step procedure with neat EDC as a coupling agent.

In a typical one-step immobilization using EDC and sulfo-NHS, magnetic PGMA-COOH or PHEMA-COOH microspheres (1 mg) were washed four times with 0.1 M phosphate buffer (pH = 7.3), magnetically separated and mixed with the following agents: EDC (7.5 mg/0.2 mL), sulfo-NHS (1.25 mg/0.2 mL), and trypsin (4 mg/0.5 mL; 0.03 wt% benzamidine). This one-step method can also be carried out without the addition of sulfo-NHS. In a two-step procedure, microspheres were first incubated in buffer with EDC (7.5 $mg \cdot mL^{-1}$) for 10 min, the supernatant with the excess of EDC was removed and trypsin solution (4 mg/0.5 mL) with benzamidine (0.03 wt%) was added to activated microspheres. For all the above-mentioned procedures the reaction proceeded in 0.1 M phosphate buffer (1 mL of total volume; pH = 7.3) at 23 °C for 3 h with gentle stirring. The trypsin-immobilized magnetic microspheres (further denoted as Tryp-PGMA-COOH or Tryp-PHEMA-COOH) were washed ten times with 0.1 M phosphate buffer (pH = 7.3) and stored at 4–8 °C in 0.1 M phosphate buffer (pH = 7.3) containing 0.05% NaN_3 solution.

Trypsin activity was determined using low-molecular-weight chromogenic substrate BApNA according to method modified from the literature.[2] Soluble or immobilized trypsin (0.1 mL) was incubated in 0.1 M NH_4HCO_3 (0.88 mL) and 0.55 M BApNA in *N,N*-dimethylformamide (0.02 mL) at room temperature for 30 min under mild stirring. The reaction was stopped by the addition of acetic acid (30 wt%, 0.2 mL) and the absorbance was measured at 405 nm.

2.7. Immobilization of huIgG on Magnetic PGMA-COOH and PHEMA-COOH Microspheres

Magnetic PGMA-COOH and PHEMA-COOH microspheres (1 mg) were washed five times with 0.1 M MES buffer (pH = 5.0) and incubated with EDC (7.5 $mg \cdot mL^{-1}$ of 0.1 M MES) for 10 min, the excess of unreacted EDC was removed from the activated microspheres by washing with 0.1 M MES buffer (2 × 1 mL) and huIgG (0.2 $mg \cdot mL^{-1}$ of 0.1 M MES) was then added. The immobilization mixture was incubated at 4–8 °C for 16 h. Subsequent washing was identical to that described above. The amount of immobilized huIgG was assessed by absorbance measurement at 260/280 nm (Eppendorf BioPhotometer; Hamburg, Germany) and by BCA test (Pierce® BCA Protein Assay Kit).

3. Results and Discussion

3.1. Synthesis and Characterization of Magnetic Macroporous Microspheres

In this report, carboxyl-terminated microspheres were preferred due to the availability of a range of standardized and optimized protocols for bioconjugating various ligands. Carboxyl groups were introduced in the microspheres by copolymerizing a relatively small amount (10 wt%) of MCMEMA (Scheme 1a) in the feed, which was subsequently hydrolyzed. The microspheres were based both on PGMA and PHEMA. PGMA has the advantage of reactive oxirane groups which can be potentially modified into any functional groups required. In addition to PGMA-COOH microspheres, PHEMA-COOH particles were fabricated as a standard, because PHEMA is commonly used in a range of bioapplications[21] due to its biocompatibility, inertness, and low-protein adsorption.[22] Generally, PGMA microspheres of narrow size distribution can be synthesized by a single-step swelling of PS template (obtained by the dispersion polymerization) with GMA followed by the polymerization.[23]

In this report, however, magnetic PGMA-COOH and PHEMA-COOH microspheres were prepared by a rather complicated multi-step swelling and polymerization method (Scheme 2). In contrast to the single-step method, cyclohexyl acetate could be used as a porogen that dissolved PS seeds. Removal of PS seeds during washing after

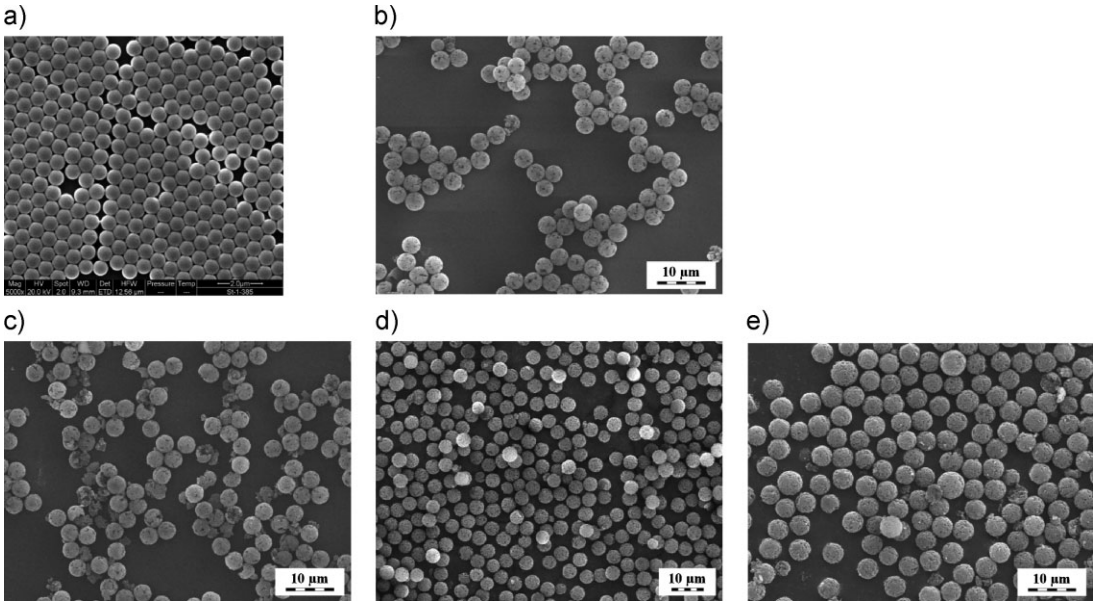


Figure 1. SEM micrographs of (a) PS seeds, (b,c) PGMA-COOH, and (d,e) PHEMA-COOH non-magnetic (b and d) and magnetic (c and e) microspheres.

completion of the polymerization thus did not induce formation of dents so typical for single-step swelling and polymerization method. Moreover, multi-step procedure produced monomer-swollen particles containing much less PS due to much higher swelling of the precursor seed than single-step swelling and polymerization could provide. The multi-step swelling and polymerization method thus produces a highly monodisperse and better quality product than other techniques. First, seeds 0.7 μm in size were prepared by emulsifier-free emulsion polymerization (Figure 1a). Second, the seeds were activated (pre-swelled) with a highly water-insoluble compound (DBP) to enable subsequent swelling with the monomers, initiator, and porogen. In the third step, DBP-swollen PS seeds 1.5 μm in diameter were swelled with the mixture of monomers, initiator, and porogen. This was followed in the fourth step by 2-hydroxyethyl cellulose- and (hydroxypropyl)methyl cellulose-stabilized and BPO-initiated suspension polymerization. In order to introduce carboxyl groups in PGMA and PHEMA microspheres, a MCMEMA comonomer (Scheme 1a) was incorporated in the polymerization feed. After its hydrolysis, [2-(methacryloyloxy)ethoxy]acetic acid (MOEAA) was obtained and 0.19 mmol of carboxyl groups was determined per g of dry PHEMA-COOH microspheres by titration with NaOH solution (Figure 2). This was less than the amount of MCMEMA in the feed (0.5 mmol), which is most likely due to the inaccessibility of some carboxyl groups buried inside the highly crosslinked polymer bulk. Nevertheless, the amount of carboxyl groups available for future modifications was sufficient according to our results. In the synthesis of PHEMA microspheres, HEMA-Ac was a monomer (Scheme 1b), which was hydrolyzed to PHEMA after the polymerization reached completion. An advantage of the HEMA-Ac monomer, compared to HEMA, is its insolubility in water, thus facilitating particle swelling and suspension polymerization in an aqueous phase. Figure 1b and d represents scanning electron micrographs (SEM) of both PGMA-COOH and PHEMA-COOH microspheres prepared by the multi-step swelling and polymerization method. The morphology of the PGMA-COOH and PHEMA-COOH particles was spherical; the particles were monodisperse (PDI = 1.04) and had diameters of 3.9 and

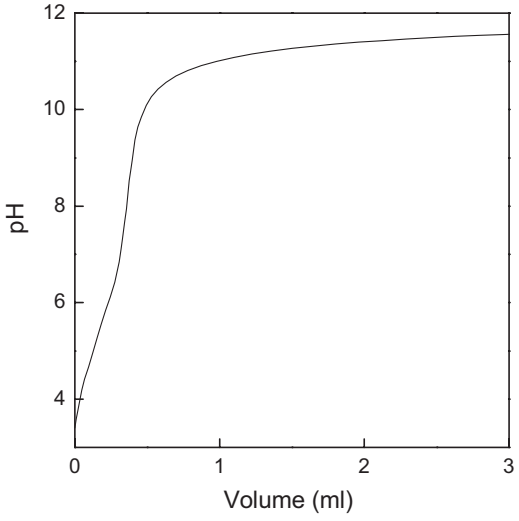


Figure 2. Titration of PHEMA-COOH microspheres (0.2 g) with 0.1 M NaOH.

4.4 μm, respectively. Their surface was rough, containing macropores formed by phase separation during the polymerization in the presence of the porogen (cyclohexyl acetate). Due to the presence of the crosslinking agent (EDMA), the specific surface area of PGMA-COOH and PHEMA-COOH particles amounted to 88.3 and 89.4 $m^2 \cdot g^{-1}$, respectively, according to the dynamic desorption of nitrogen. PGMA microspheres contained oxirane groups, which made additional functionalization possible, resulting in a range of various reactive groups.[24] In this study, oxirane groups were transformed into hydroxyl groups by hydrolysis, thus increasing the hydrophilicity of the product, which is important in reducing non-specific protein adsorption. This was confirmed by attenuation total Fourier-transform reflectance infrared (ATR-FTIR) spectra of initial PGMA-COOH microspheres with the typical transmission peak of oxirane groups at 910 cm^{-1}, which disappeared after the hydrolysis (Figure 3). Moreover, the transmission peak in the region of O–H stretching vibration (≈3400 cm^{-1}) was higher after hydrolysis, signifying that the number of hydroxyl groups had increased.

Both PGMA and PHEMA hydrogels are known to absorb large amounts of water but remain both insoluble and biologically, chemically, and mechanically stable, preserving their shape.[25] The equilibrium water uptake of PGMA-COOH and PHEMA-COOH microspheres was 2.7 and 2.8 $mL \cdot g^{-1}$, respectively, thus reflecting the hydrophilicity of the particles.

To produce magnetic carriers, maghemite (γ-Fe_2O_3) was prepared inside the pores of the macroporous microspheres by a two-step oxidation of $Fe(OH)_2$ with oxygen and sodium hypochlorite (Scheme 2). First, magnetite (Fe_3O_4) was formed by the precipitation of a ferrous salt with ammonium hydroxide and oxidation with oxygen. Second, magnetite was then oxidized with sodium hypochlorite, yielding chemically more stable γ-Fe_2O_3 (maghemite).[26] Neither the morphology nor size of the PGMA-COOH or PHEMA-COOH microspheres substantially changed after the precipitation of iron oxide inside the microsphere pores (Figure 1c and e). Magnetic PGMA-COOH and PHEMA-COOH microspheres contained 13.2 and 19.4 wt% Fe, respectively, according to atomic absorption spectrometry (AAS). This amount of Fe in the microspheres is sufficient to respond quickly to a magnetic field as was confirmed by earlier measurements of magnetic properties of analogous PGMA microspheres (prepared by the dispersion polymerization) with a magnetometer.[27] The advantage of maghemite over magnetite is its oxidation stability. Due to its low solubility in water and ability to form coordination complexes with carboxyl groups, it was not released from the microspheres in aqueous media.

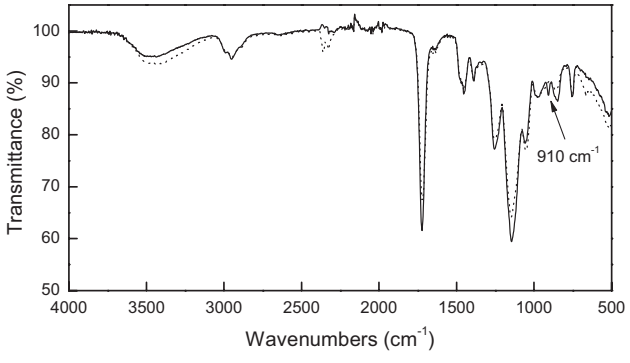


Figure 3. ATR-FTIR spectra of PGMA-COOH microspheres (—) before and (···) after hydrolysis with H_2SO_4.

3.2. Biofunctionalization of Magnetic PGMA-COOH and PHEMA-COOH Microspheres

Trypsin was immobilized on magnetic PGMA-COOH and PHEMA-COOH microspheres via EDC/sulfo-NHS chemistry. The amount of immobilized trypsin was subsequently measured by colorimetric assay with a chromogenic substrate. This approach enables the amount of actually active enzyme molecules to be quantified. Benzamidine is a low-molecular-weight competitive inhibitor of trypsin, which prevents the self-cleavage of trypsin during the immobilization process. Its presence in the immobilization mixture enhances the binding efficiency of trypsin. After the binding procedure, benzamidine is removed by simple washing.

For PGMA-COOH microspheres, the highest activity of immobilized trypsin was achieved with a one-step immobilization using EDC/sulfo-NHS at 23 °C for 3 h (Figure 4). The same trend was observed for magnetic PHEMA-COOH microspheres.

Figure 5 shows the effect of increasing amounts of trypsin in the binding mixture on the amount of enzyme

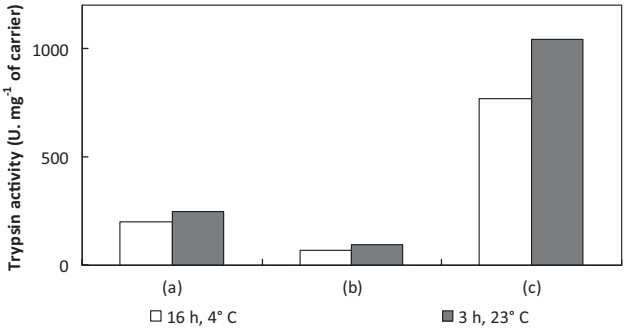


Figure 4. Proteolytic activity of magnetic Tryp-PGMA-COOH microspheres under various reaction conditions (time, temperature, and presence of EDC and sulfo-NHS). (a) One-, (b) two-step protocol with EDC, and (c) one-step immobilization with EDC and sulfo-NHS.

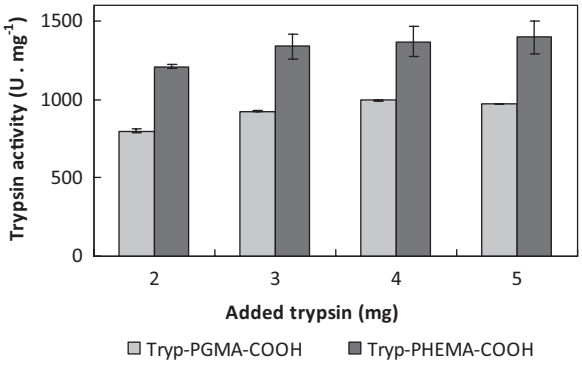


Figure 5. Determination of proper amount of trypsin in binding mixture per mg of magnetic microspheres.

which was immobilized onto the microspheres and simultaneously active. In both types of magnetic carriers, a slight increase in activity was observed as a consequence of increasing the trypsin content in the binding mixture. The optimal amount was found to be 4 mg of trypsin per mg of both magnetic microspheres. Immobilizing higher amounts of enzyme (>5 mg) was not beneficial; the immobilization efficiency slightly decreased (Figure 5).

The average enzyme activity of magnetic Tryp-PGMA-COOH and Tryp-PHEMA-COOH microspheres determined from three repetitions was 1021 ± 44 and $1418 \pm 32\ U \cdot mg^{-1}$ of microspheres, respectively.

To verify that the enzyme molecules were specifically adsorbed, they were washed with 1 M NaCl solution. Enzyme activity then slightly decreased to 99.8 and 99.3% for magnetic Tryp-PGMA-COOH and Tryp-PHEMA-COOH microspheres, respectively. The above-mentioned reduction of trypsin activity is statistically insignificant; hence, the results indicate that more than 99% of the trypsin was coupled to the microspheres through covalent bonds.

Based on good experience with the simultaneous co-immobilization of proteins and other ligands to microspheres, and keeping in mind the aim of covering these hydrophobic clusters, which are responsible for the non-specific adsorption of biomolecules from complex biological mixtures, the addition of inert protein – BSA – could increase the quality of enzyme-biofunctionalized microspheres.[28] BSA solution (33 wt%) was added to the immobilization mixture 10 min after the start of the immobilization. This was followed by a 16-h incubation and subsequent washing with 0.1 M phosphate buffer (pH = 7.3) containing 1 M NaCl for the elimination of eventual non-specifically bound molecules. Magnetic Tryp-PGMA-COOH microspheres achieved a trypsin activity comparable to the procedure without albumin addition (difference <5%).

The chemical as well as biological stability of these newly developed biofunctionalized magnetic microspheres needs to be confirmed before their routine application. Operational and storage stability were therefore verified. The activity of magnetic Tryp-PGMA-COOH and magnetic Tryp-PHEMA-COOH microspheres was repeatedly determined in nine cycles within 1 d (0.1 mg aliquot, using the same one each time). The activity of magnetic Tryp-PHEMA-COOH microspheres decreased only moderately to 60% in the fifth cycle. The activity of magnetic Tryp-PGMA-COOH microspheres decreased more considerably, reaching 39.6% of the original value in the fifth cycle. These results were not what we expected; previous results showed that >50% of the original activity was retained even after the 10th measurement.[2] One of several reasons for the activity decrease could be the absence of BSA during immobilization or the autoproteolytic behavior of trypsin. This issue still needs to be studied in more detail. Storage stability tests, i.e. determination of enzyme activity over one-week periods (0.1 mg aliquot, a fresh one each time) gave excellent results and confirmed the suitability of the prepared biofunctionalized carriers for long-term storage as well as their applicability. More than 70% of the trypsin molecules immobilized on Tryp-microspheres were active after seven weeks of storage, which fulfilled our expectations (Figure 6).

According to the literature, zeta-potential measurements of free and enzyme-modified carriers correlated with enzyme binding efficiency.[29] Thus, we measured the zeta-potential of trypsin-free and trypsin-modified magnetic microspheres in various buffers at various molar concentrations (0.01, 0.05, and 0.1 M). The absolute values of zeta-potential of the microspheres in phosphate buffer decreased after their biofunctionalization with trypsin (Figure 7). A similar tendency was also observed for measurements of the zeta-potential of magnetic Tryp-PGMA-COOH and Tryp-PHEMA-COOH microspheres in NH_4HCO_3 buffer. The lower molar concentrations of buffer

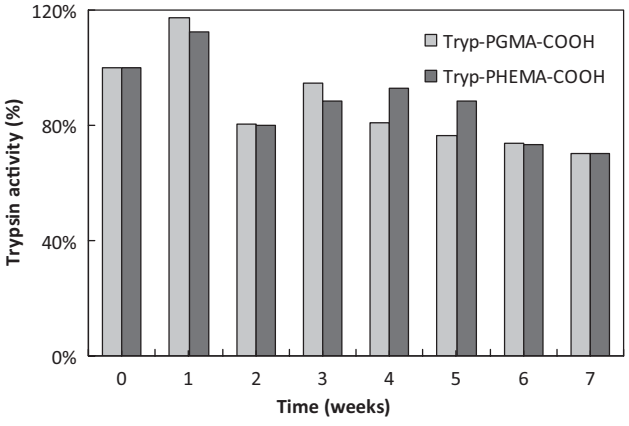


Figure 6. Storage stability of magnetic Tryp-PGMA-COOH and magnetic Tryp-PHEMA-COOH microspheres at 4 °C in 0.1 M phosphate buffer (pH = 7.3) containing 0.03 wt% benzamidine (0.1 mg aliquots).

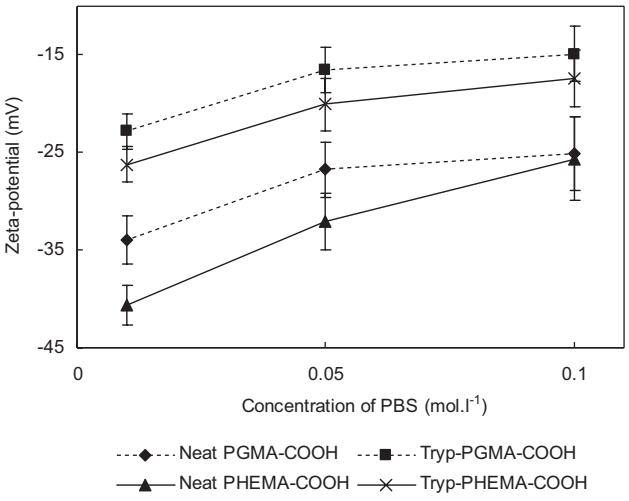


Figure 7. Zeta-potential of magnetic PGMA-COOH and magnetic PHEMA-COOH microspheres before and after biofunctionalization with trypsin in phosphate buffer (pH = 7.3); activity 970 U · mg^{-1} of Tryp-PGMA-COOH and 1417 U · mg^{-1} of Tryp-PHEMA-COOH.

were used, the higher absolute values of zeta-potential of carriers were measured indicating better colloidal stability and lower rate of aggregation due to the repulsion among individual particles. Based on these results, the buffers with a lower molar concentration are preferred for this type of microspheres.

Regarding the potential use of microspheres as carriers of antibodies in the field of immunoprecipitation, human IgG was immobilized on both types of new magnetic microspheres. Based on earlier investigations,[6] an optimized two-step carbodiimide method was chosen for covalently binding huIgG to the microspheres. Two different approaches were used for determining the amount of immobilized huIgG on the microspheres. The total amount of immobilized huIgG was calculated as the difference between the amount of IgG in the binding solution before immobilization and the sum of IgG from all solutions after immobilization, measured by UV spectrophotometry at 260/280 nm. Additionally, the BCA test with IgG-immobilized microspheres was done according to the manufactures' instructions. For both methods, the amount of immobilized IgG was calculated for 1 mg of carrier. Both methods confirmed that the IgG molecules were successfully immobilized onto magnetic PGMA-COOH (76.7 μg of huIgG per mg) and PHEMA-COOH microspheres (124.2 μg of huIgG per mg).

To summarize, both of the newly developed magnetic PGMA-COOH and PHEMA-COOH microspheres presented here can be recommended as suitable carriers for enzyme and also antibodies immobilization.

4. Conclusion

Magnetic monodisperse PGMA-COOH and PHEMA-COOH microspheres were obtained by the multi-step swelling and polymerization method followed by the precipitation of iron oxide inside the pores of the particles. To verify the applicability of the developed magnetic PGMA-COOH and PHEMA-COOH microspheres for immobilizing biocompounds, the proteolytic enzyme trypsin and human IgG were used as model ligands. Trypsin was immobilized on the microsphere surface by the well-known carbodiimide-mediated one-step protocol with heterobifunctional cross-linker EDC and sulfo-NHS agent, and strong bond creation was confirmed. These newly developed biofunctionalized magnetic microspheres demonstrated the possibility of long-term storage without significant changes, which thus indicates great potential for their successful use in bioapplications. Also, IgG-bionfunctionalized microspheres have been shown to be convenient carriers for, e.g., widely used immunoprecipitations.

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