DOI: 10.1016/j.msec.2014.04.011

PEGylation of magnetic poly(glycidyl methacrylate) microparticles for microfluidic bioassays

Jana Kucerova[a], Zuzana Svobodova[a], Petr Knotek[b], Jiri Palarcik[c], Milan Vlcek[d1], Miloslav Kincl[d1], Daniel Horak[d], Julien Autebert[e], Jean Louis Viovy[e], Zuzana Bilkova[a*]

[a] Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 10 Pardubice, Czech Republic.

[b] Joint Laboratory of Solid State Chemistry of IMC and University of Pardubice, Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 10 Pardubice, Czech Republic.

[c] Institute of Environmental and Chemical Engineering, Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 10 Pardubice, Czech Republic.

[d] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic.

[e] Macromolecules and Microsystems in Biology and Medicine, Institute Curie, UMR 168, 26 Rue d′Ulm, 75005 Paris, France.

[1] Present address: Joint Laboratory of Solid State Chemistry of Institute of Macromolecular Chemistry of the Academy of Sciences of the Czech Republic and University of Pardubice, Studentska 84, 532 10 Pardubice, Czech Republic.

*Corresponding author: Assoc. Prof. RNDr. Zuzana Bilkova, Ph.D., Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentska 573, 532 10 Pardubice, Czech Republic, zuzana.bilkova@upce.cz, Tel.: +420 466 037 700, fax: +420 466 037 068.

Abbreviations: PEG, poly(ethylene glycol); PGMA-COOH microspheres, poly(glycidyl methacrylate) microspheres containing carboxyl groups; CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres, poly(glycidyl methacrylate) microspheres coated by CH_3-$PEG_{30,000}$-NH_2 (Mr of 30,000 Da); CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres, poly(glycidyl methacrylate) microspheres coated by CH_3-$PEG_{2,000}$-NH_2 (Mr of 2,000 Da); HO-$PEG_{3,400}$-NH_2-PGMA microspheres, poly(glycidyl methacrylate) microspheres coated by HO-$PEG_{3,400}$-NH_2 (Mr of 3,400 Da); biotin-$PEG_{2,000}$-NH_2-PGMA microspheres, poly(glycidyl methacrylate) microspheres coated by biotin-$PEG_{2,000}$-NH_2 (Mr of 2,000 Da); BSA, bovine serum albumin; HRP, horseradish peroxidase; PDMS, poly(dimethylsiloxane); COC, cyclic olefin copolymer; ZP, zeta potential; pI, isoelectric point; NSA, nonspecific adsorption; PBS, phosphate buffered saline; SEM, scanning electron microscopy; SD, standard deviation.

Keywords

PEGylation; magnetic microspheres; microfluidics; nonspecific adsorption

Abstract

In this study, magnetic poly(glycidyl methacrylate) microparticles containing carboxyl groups (PGMA-COOH) were coated using highly hydrophilic polymer poly(ethylene glycol) (PEG). PEG was used to reduce nonspecific interactions with proteins and cells while decreasing adhesion of particles to the walls of a microfluidic devices from poly(dimethylsiloxane) (PDMS) and cyclic olefin copolymer (COC). Zeta potential measurement, infrared spectroscopy, scanning electron microscopy, anti-PEG ELISA assay, and bioaffinity interactions between biotin and streptavidin-HRP successfully proved the presence of PEG on the surface of microspheres. Both neat and PEGylated microspheres were then incubated with the inert protein bovine serum albumin or cells to evaluate the rate of nonspecific adsorption (NSA). PEG with Mr of 30,000 Da was responsible for 45% reduction in NSA of proteins and 74% for cells compared to neat particles. The microspheres' behavior in PDMS and COC microchannels was then evaluated. Aggregation and adhesion of PEGylated microspheres significantly decreased compared to neat particles. Finally, the model enzyme horseradish peroxidase was immobilized on the microspheres through the heterobifunctional PEG chain. The possibility for subsequent covalent coupling of the ligand of interest was confirmed. Such PEGylated microparticles can be efficiently used in PDMS microchips as a carrier for bioaffinity separation or of enzyme for catalysis.

1. Introduction

Recently, superparamagnetic particles have been put to use across a broad spectrum of both scientific and industrial fields and applications. In biomedicine, they have found application particularly in *in vitro* diagnostics (immunoassays, manipulation and separation of cells and nucleic acids), *in vivo* diagnostics (magnetic resonance imaging), and therapies (drug and gene delivery, hyperthermia) [1-5]. Their easy manipulation, potential for automation, and large surface-to-volume ratios make them very attractive for such purposes.

The boom in microfluidics, which began about 20 years ago, brought with it ideas about incorporating magnetic particles into various microfluidic devices [6-9]. This offers additional benefits compared to batch-type processes, such as reduced sample and reagent volumes, faster reaction times, the possibility to engineer the design of a chip to control particle mobility, and integration of various processes into a unified system [5]. Even in such a well-described area, however, problems can arise in connection with particles adhering onto inner walls of microchannels and tubing accompanied by nonspecific adsorption (NSA) of proteins or cells. These complications are amplified when working with a complex biological material like whole blood or serum. Two major strategies for minimizing these shortcomings have been investigated. They utilize surface modification either to the inner walls of microfluidic devices or to the

particles themselves. The first approach includes grafting inner walls of microfluidic channels with hydrophilic compounds and developing suitable polymers for chip manufacture, dynamic coatings, and self-assembled monolayers [10-13]. The second approach, described herein, includes surface modification of magnetic particles using highly hydrophilic polymers before their introduction into the chip.

For surface modification of particles, synthetic polymers or copolymers, such as poly(ethylene glycol) (PEG), polyacrylamide, polyethylene, poly(ε-caprolactone), poly(vinyl alcohol), or such natural macromolecules as dextran and chitosan are widely used, as reviewed by Audonnet et al. [14] . Among these approaches, PEGylation is the most accepted tool for increasing the biocompatibility and reducing undesirable nonspecific interactions with biomolecules, and particularly proteins or cells [15-17]. Furthermore, PEG improves colloidal stability of the particles and can serve as a spacer for subsequent immobilization of biologically active ligands (enzymes, antibodies, etc.). Due to its low toxicity, PEG is highly suitable for biomedical applications [15]. Unlike methods for PEG coupling, which have been thoroughly examined and described [18-20], there exists a limited choice of methods for detecting PEG on the surface of spherical magnetic microparticles.

Numerous methods have been developed for determining the extent to which proteins have been modified by PEG. For example, conventional colorimetric (TNBS, fluorometric) [21, 22] and chromatographic characterization (HPLC-SEC, RP-HPLC, gel permeation chromatography) [23-25] in combination with light scattering, MALDI-MS [24, 26-28], and capillary electrophoresis [29, 30] have been applied for analysis of PEGylated proteins. Specific colorimetric detection of PEG with iodine [31, 32] using Fourier transform infrared (FTIR) or Raman spectroscopy [24] and nuclear magnetic resonance [33, 34] have also been described. To monitor flat PEGylated surfaces, methods typically used include contact angle and zeta potential measurements, X-ray photoelectron spectroscopy (XPS), ellipsometry, as well as atomic force (AFM), scanning electron (SEM) and transmission electron (TEM) microscopy [16]. PEGylation has been commonly proven by adsorption studies using model proteins, where the PEG provided good resistance against NSA [35]. Nevertheless, detecting PEG on the surfaces of magnetic particles, and especially of those with sizes of several micrometers, remains challenging due to the particles' spherical shape and rapid sedimentation caused by the presence of iron oxides that increase the density of such microspheres.

The aims of this study were to prepare magnetic polymer microparticles with enhanced nonfouling surfaces, then to characterize and verify their properties as required for implementation in microfluidic chips. These properties include (i) minimal NSA of molecules from complex biological mixtures, (ii) stability against aggregation of particles under various conditions, (iii) minimal adhesion of particles on inner walls of microfluidic devices and tubes, and (iv) satisfactory magnetic response when inserted into a

magnetic field. In this study, new poly(glycidyl methacrylate) microspheres containing carboxyl groups (PGMA-COOH) were prepared by a multistep swelling and polymerization [36] originally developed for polystyrene microspheres [37]. The particles were subsequently functionalized by PEG. A variety of methods based on different principles were used to monitor the presence of PEG on the surface of the particles. Antifouling properties of PEGylated particles were compared with those of the neat particles. Their behavior was also compared when inserted into microfluidic channels, where the adhesion of particles was observed. Covalent coupling of horseradish peroxidase showed the possibility for subsequent attachment of a biologically active ligand of interest. Here, the widely applied PEGylation was newly used as a tool to improve the characteristics of magnetic microparticles associated with their behavior in PDMS and COC microfluidics chips.

2. Materials and methods

2.1. *Materials*

Monofunctional CH_3-$PEG_{30,000}$-NH_2 (Mr 30,000), heterobifunctional biotin-$PEG_{2,000}$-NH_2 (Mr 2,000), and NH_2-$PEG_{3,400}$-OH (Mr 3,400) from Laysan (Laysan Bio, Arab, AL, USA) were used for PEGylation. CH_3-$PEG_{2,000}$-NH_2 (Mr 2,000), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysulfosuccinimide salt (sulfo-NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES), bovine serum albumin (BSA), streptavidin-horseradish peroxidase (streptavidin-HRP), sodium periodate ($NaIO_4$), horseradish peroxidase (HRP; 300 units/mg), sodium cyanoborohydride, and *o*-phenylene diamine were purchased from Sigma Aldrich (St. Louis, MO, USA). The Thermo Scientific Pierce BCA Protein Assay Kit was acquired from Thermo Fisher Scientific (Rockford, IL, USA). The PEGylated protein ELISA kit was from Enzo Life Sciences (Farmingdale, NY, USA). Other chemicals were of analytical grade and obtained from Penta (Chrudim, Czech Republic) or Lach-Ner (Neratovice, Czech Republic). All buffers and solutions were prepared from ultrapure water filtered through a TKA Smart2Pure system (Thermo Scientific TKA, Germany). MCF7 cell line was purchased from Health Protection Agency Culture Collections (Salisbury, UK). Sera-Mag Double Speed Magnetic Carboxylate-Modified Microparticles (further denoted as Sera-Mag-Carboxyl) were obtained from Seradyn (Indianapolis, IN, USA), SiMAG-Carboxyl particles were from Chemicell (Berlin, Germany), and Dynabeads M-270 Carboxylated were from Life Technologies (Carlsbad, CA, USA).

2.2. Synthesis of magnetic poly(glycidyl methacrylate) particles with carboxyl groups

Magnetic poly(glycidyl methacrylate) (PGMA) microspheres containing carboxyl groups (PGMA-COOH) were prepared according to a previously described procedure [36] by multistep swelling and a polymerization method based on polystyrene latex seeds. The seeds were activated with dibutyl phthalate to enable subsequent swelling with monomers (glycidyl methacrylate, ethylene dimethacrylate, and [2-

(methacryloyloxy)ethoxy]acetic acid) and a porogen (cyclohexyl acetate). In order to produce polymer particles, suspension polymerization of the swollen seeds, which was stabilized by (hydroxypropyl) methyl cellulose and initiated by benzoyl peroxide, was carried out at 70 °C for 16 h. The resulting macroporous particles were used as a base for repeated precipitation of iron oxides using ammonia and air oxygen from $FeCl_2$ solution imbibed within the particles' pores. The iron content was analyzed by atomic absorption spectrometry (AAS Perkin-Elmer 3110, Perkin-Elmer, Waltham, MA, USA).

2.3. PEGylation of microspheres

PEGylation was performed by two-step carbodiimide coupling in the presence of sulfo-NHS reagent [7, 38]. EDC and sulfo-NHS in 0.01 M MES buffer (pH 5) were added to 1 mg of washed microspheres in final concentrations of 0.0391 M and 0.0057 M, respectively. The activation process occurred at room temperature (RT) for 5 min under stirring. The required amount of PEG (biotin-$PEG_{2,000}$-NH_2, HO-$PEG_{3,400}$-NH_2, CH_3-$PEG_{30,000}$-NH_2, or CH_3-$PEG_{2,000}$-NH_2) in 0.01 M MES buffer (pH 5) was added to a suspension of the microspheres. The amount of PEG to be immobilized was set as 2.5 fold molar excess relative to carboxyl group content on the microsphere surface. Immobilization proceeded at 4 °C overnight under mild stirring on a rotator. The PEGylated microspheres were washed seven times with 1 mL of 0.1 M MES (pH 5), once with 1 mL of 0.1 M MES (pH 5) containing 1 M NaCl, and two times with 1 mL of 0.1 M MES (pH 5).

2.4. Analysis of PEGylated microspheres

2.4.1.Zeta potential measurement

Zeta potential (ZP) of neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA was measured in 0.01 M KCl and 0.01 M phosphate buffer (pH 7.0) using a ZetaPALS apparatus (Brookhaven Instruments; Holtsville, NY, USA). The microspheres concentration was 0.05 mg per 1 mL of solution and total volume was 1.5 mL. Each measurement incorporated at least 6 cycles with 10 single measurements and several stirring steps to avoid sedimentation of the microspheres. The measured values were averaged and standard deviation (SD) calculated.

2.4.2.Isoelectric point assessment

For determining isoelectric point (pI) of the microspheres, 0.5 mg of PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA were resuspended in 11 mL of ultrapure water and pI was assessed using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, United Kingdom) with the addition of 0.25 M HCl or NaOH, respectively.

2.4.3.Scanning electron microscopy and image analysis

Neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres were prepared for SEM imaging by spin-coating. Images of gold-coated samples were acquired using a JEOL JSM-5500LV microscope (Tokyo, Japan). The diameter (with standard error of measurement ± 50 nm) was determined as the average of two perpendicular diameters of each microsphere at full width at half maxima [39, 40] for number of particles > 30 employing Gwyddion software version 2.25 [41].

2.4.4.Infrared (IR) spectroscopy

Infrared diffuse reflectance spectra were measured with the FTIR Thermo Nicolet Nexus (Waltham, MA, USA) spectrophotometer in the range 4000–400 cm^{-1} with resolution of 2 cm^{-1} using a dry KBr powder containing 1% by weight of the polymer. The number of scans was 128 at the mirror velocity 0.158 cm s^{-1}. The diffuse reflection spectra (R) thus obtained were converted to absorption spectra using the Kubelka–Munk (KM) function as $KM(R) = (1 - R)^2/(2R)$ [42]

2.4.5.Biotin-streptavidin assay

To determine maximum binding capacity of the PGMA-COOH microspheres, particles were coated with an increasing amount of biotin-$PEG_{2,000}$-NH_2 (0.0005–1 mg). To compare binding capacity between PGMA-COOH microspheres and commercial particles (Sera-Mag-Carboxyl, SiMAG-Carboxyl, and Dynabeads M-270 Carboxylated), a standard quantity of biotin-$PEG_{2,000}$-NH_2 as mentioned in the section 2.3 was used for immobilization. A total of 0.1 mg of microspheres was then resuspended in 0.01 mL of 0.1 M phosphate buffer (pH 6.2). Streptavidin-HRP conjugate (0.09 mg) dissolved in 0.09 mL of 0.1 M phosphate buffer (pH 6.2) was added and the mixture incubated for 30 min under stirring. This was followed by nine washing steps with 0.1 M phosphate buffer (pH 6.2) and one washing step with the same buffer containing 1 M NaCl. The activity of HRP was determined on 0.03 mg of microspheres as described elsewhere [43]. The absorbance at 495 nm was measured using a PowerWave 340 Microplate Spectrophotometer (Biotek Instruments, Winooski, VT, USA). Each measurement was repeated three times. To determine maximum binding capacity, values were related to absorbance of the maximum tested quantity (set at 1 mg of biotin-$PEG_{2,000}$-NH_2). The values thus obtained were plotted according to the amount of biotin-$PEG_{2,000}$-NH_2 added in the reaction. All measurements were repeated a minimum of three times, the values averaged and SD shown in graph.

2.4.6.PEGylated protein ELISA kit

The commercial kit originally intended for analysis of PEGylated proteins was used to prove the presence of PEG on the surface of CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres. The assay is based on competitive ELISA, where the monoclonal anti-PEG antibodies are immobilized on the surface of microwells. In total, 0.05 mg of neat PGMA-COOH and 0.05 mg of CH_3-$PEG_{30,000}$-NH_2-PGMA

microspheres were transferred into wells of a microtiter plate. The procedure was conducted and data calculated according to the manufacturer's instructions. Measurements were repeated three times, the values averaged and SD shown in graph.

2.5. Evaluation of behavior of PEGylated microspheres and nonspecific adsorption

2.5.1.Evaluation of NSA of proteins

A total of 0.5 mL of the 10% w/v solution of BSA was added to the 0.5 mg of neat PGMA-COOH, CH_3-$PEG_{30,000}$-NH_2-PGMA, and CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres. After 60 min of incubation (mild stirring, RT), the BSA content in supernatants was quantified using a Thermo Scientific Pierce BCA Protein Assay Kit. Measurements were repeated four times, the values averaged and SD shown in graph.

2.5.2.Evaluation of NSA of cells

The MCF7 cell line was cultured in Dulbecco modified Eagle's minimal essential medium supplemented with aqueous penicillin/streptomycin (100 μg/mL), 10% fetal bovine serum and insulin (0.01 mg/mL) at 37 °C in a humidified, 5% CO_2 atmosphere. The magnetic microspheres (neat PGMA-COOH, CH_3-$PEG_{30,000}$-NH_2-PGMA, and CH_3-$PEG_{2,000}$-NH_2-PGMA), totaling 0.5 mg per tube, were washed three times with 1 mL of PBS (pH 7.4) containing 0.1% BSA using a magnetic separator. The particles were mixed with 2×10^6 of cells and rolled in a tube rotator at RT for 1 h. In the next step, the microspheres with isolated cells were washed five times with PBS (pH 7.4) containing 0.1% BSA to remove all unbounded cells, always using a new Eppendorf tube. The nonspecifically captured cells were observed and counted in a Bürker's chamber using the Nikon Eclipse 80i microscope (Tokyo, Japan) and a Nikon digital sight DS-MS camera. The images were processed using NIS-Elements AR Analysis 3.2 software (Nikon, Tokyo, Japan). The experiment was repeated three times, the values averaged and SD shown in graph.

2.5.3.Evaluation of adhesion on the microfluidic chips

To test the behavior of particles in chip, a simple PDMS device containing seven straight channels (Fig. 1) with a rectangular cross-section was fabricated using soft lithography [44] according to a procedure previously described [45]. The inner dimensions of each channel were (w x h x l): 200 μm x 50 μm x 30 mm. Hamilton syringes (Bonaduz, Switzerland) and PM-1000 syringe pump (Protea Biosciences, Langlade, France) were used in this experiment. Channels of the chip were coated prior to use with a copolymer of dimethylacrylamide and allyl glycidyl ether[46] at a concentration of 1 wt%, during 30 min. Channels were then rinsed with 0.175 mL of PBS (pH 7.4) containing 0.1% BSA. Suspension of microspheres (neat PGMA-COOH, CH_3-$PEG_{30,000}$-NH_2-PGMA, or CH_3-$PEG_{2,000}$-NH_2-PGMA) of known concentration (1 mg per 0.05 mL of PBS pH 7.4, accurate number of particles was counted in a Bürker's chamber) was then injected into the channel (flow rate 0.1 mL/h). The flow was subsequently stopped for

10 min to allow the microspheres to interact with the surface of the channel. This was followed by rinsing (0.2 mL, flow rate 0.5 mL/h) and cleaning steps (0.3 mL, flow rate 0.5 mL/h) with PBS (pH 7.4) containing 0.1% BSA. The entire experiment was monitored using a USB Dino-Lite 7013/7023 microscope and Dino-Capture software (AnMo Electronics, Taipei, Taiwan). The number of particles adhered on the walls of channel was counted.

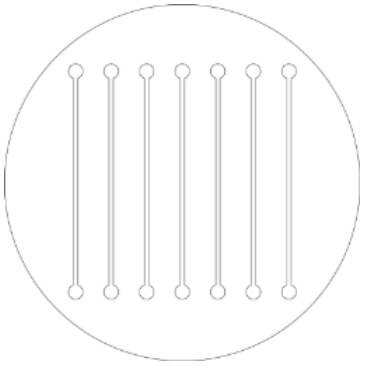

Fig. 1: A sketch of PDMS chip used for evaluation of the adhesion of neat and PEGylated microspheres.

Also, the commercial microfluidic chips made from COC (topas) with hydrophilized channels and rhombic chambers eP1 (the channel dimensions (w x h x l): 650 µm x 500 µm x 32.5 mm) from microfluidic ChipShop (Jena, Germany) were applied to test the particles adhesion on the inner surface of the channels. The channel of the chip was rinsed and the particles were injected as described in the previous paragraph. The ChipGenie edition P instrument was used for the whole experiment. After 10 min of particles homogenization, images of the suspension were taken using USB-Dino Lite microscope (see above) and the volume of the plug was measured using the free software Image J (National Institute of Mental Health, Bethesda, MD,USA).

2.6. Immobilization of horseradish peroxidase on the PEGylated microspheres and determination of HRP activity

First, heterobifunctional HO-$PEG_{3,400}$-NH_2 was immobilized on the PGMA-COOH microspheres according to the procedure reported above (see 2.3). The immobilization of HRP occurred through the terminal hydroxyl groups of PEG, as described elsewhere [47]. Briefly, hydroxyl groups of the HO-$PEG_{3,400}$-NH_2-PGMA microspheres (1 mg) were oxidized during 90 min incubation with 0.1 M sodium periodate. Five washing steps with 0.1 M phosphate buffer (pH 7.3) followed. HRP (2 mg in 0.9 mL) was added to the microspheres. After 10 min of incubation, 3 mg of sodium cyanoborohydride were added and immobilization proceeded overnight at 4 °C. The microspheres were washed ten times with 0.1 M phosphate buffer (pH 7.3). The activity of HRP was determined using hydrogen peroxide as a substrate and *o*-phenylene diamine as a chromogen [43], and the absorbance at 495 nm was measured.

3. Results and discussion

3.1. Synthesis of magnetic monodisperse PGMA-COOH microspheres and their PEGylation

The particles were obtained by multistep swelling and polymerization [36]. The procedures began from monodisperse 0.7 μm polystyrene seeds. In order to produce final PGMA particles with ~4 μm size, the volume of the seeds had to be increased by approximately 200 times. Swelling of seeds therefore proceeded in two steps: (i) with a highly water-insoluble compound (dibutyl phthalate) to facilitate (ii) swelling with monomers and porogen. In addition to GMA, [2-(methacryloyloxy)ethoxy]acetic acid and ethylene dimethacrylate formed the polymerization feed. While the [2-(methacryloyloxy)ethoxy]acetic acid provided carboxyl groups (~0.5 mmol COOH/g), the use of ethylene dimethacrylate as a crosslinking agent ensured good mechanical properties of the particles and their insolubility in media. At the same time, it enabled formation of porous structure in the presence of porogen (cyclohexyl acetate) in the feed. Swelling was performed in several steps in order to maintain monodispersity of the initial polystyrene seeds. After completion of the polymerization, porosity provided space for precipitation of magnetic iron oxides inside the particles to render them with magnetic properties. The PGMA-COOH microspheres contained 23.8% by weight Fe and they were quickly attracted by a magnet.

The PGMA-COOH microspheres had been previously proven successful in immobilizing the model enzyme trypsin as well as human immunoglobulin G [36]. Nevertheless, high NSA and adhesion were observed after their biofunctionalization with specific antibodies and immunocapture in the PDMS microchannel. Additional surface modification of the microspheres was thus required to minimize nonspecific interactions during the analysis of complex biological material. PEGylation was therefore selected as a promising tool to overcome the aforementioned shortcomings.

There exist several strategies for introducing PEG onto the surface of particles, namely, physisorption, "grafting to" and "grafting from" [14, 15]. Although the "grafting from" strategy enables immobilization of PEG with high grafting density, the "grafting to" approach was chosen for this study as it is less elaborate and time-consuming while providing complete coating of the microspheres. As the microspheres are used in microfluidic devices and the coating must be mechanically resistant and stable, immobilization through strong covalent bonding was preferred. The PEGylation was mediated by carbodiimide chemistry using EDC and sulfo-NHS as zero-length crosslinkers. In this method, carboxyl groups on the microspheres were first activated using water-soluble EDC and sulfo-NHS to form an intermediate, which subsequently reacted with the amine groups of the PEG chains [48]. When PEGs with both reactive terminal groups were immobilized (e.g., HO-PEG-NH_2), there was a risk of EDC-mediated polymerization of PEG chains. A two-step protocol for immobilization of PEG was therefore chosen to avoid this unwanted crosslinking.

3.2. Analysis of PEGylated microspheres

It is generally difficult to confirm a presence of PEG on the surface of the magnetic particles due to their size, shape, and the presence of iron ions. Methods like dynamic light scattering, contact angle measurements or nuclear magnetic resonance have proven unsuitable for analysis of PEGylated PGMA-COOH microspheres. Zeta potential (ZP) and isoelectric point (pI) measurement, SEM accompanied by image analysis, IR spectroscopy, biotin-streptavidin based interactions, and anti-PEG-ELISA were thus used for characterizing the particles in this work.

Measured ZP of neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres clearly demonstrated that a presence of PEG chains significantly affected the ZP values (Fig. 2). As a result of PEGylation, ZP increased in both tested solutions. The difference in ZP before and after PEG-coating reached 13 mV in KCl and 14 mV in phosphate buffer, which is probably caused by the presence of a methoxy group at the end of the PEG. The results correspond with those in previously published reports [49, 50] and proved that PEG was present on the microspheres. In addition to ZP, pI values of both neat and PEGylated PGMA microspheres were determined. For neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA particles, the pI was 2.9 and 3.34, respectively. The appropriate pH for working with CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres from the viewpoint of particles' stability was shown to be >6, which is close to the physiological pH and suitable for the desired bioapplications.

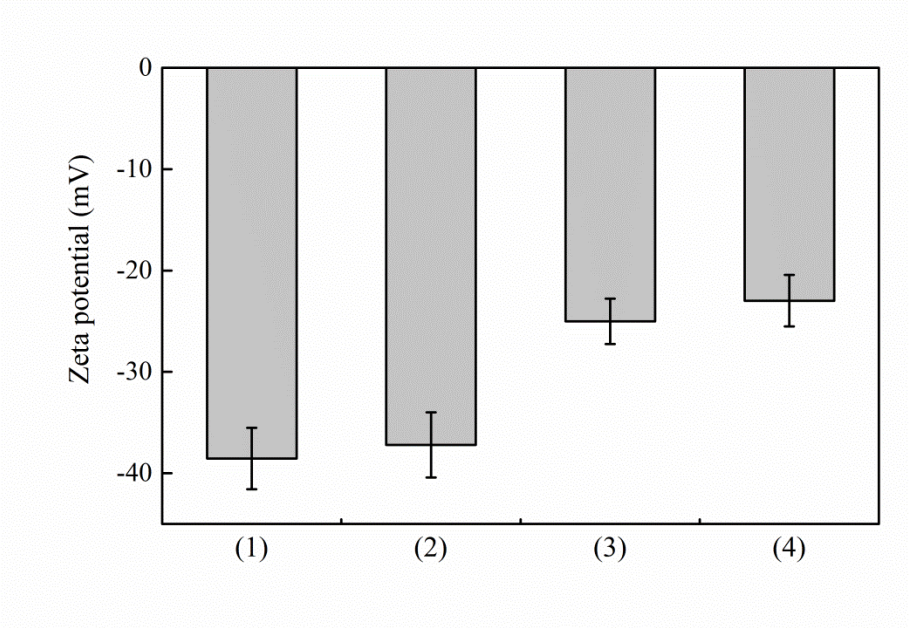

Fig. 2. Zeta potential of neat PGMA-COOH microspheres in (1) 10^{-3} M phosphate buffer and (2) 10^{-3} M KCl solution as well as zeta potential of CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres in (3) 10^{-3} M phosphate buffer and (4) 10^{-3} M KCl solution.

The increase in microsphere diameters induced by PEG-coating was also detected by SEM. The diameters of the neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres were semi-statistically determined by analysis of the SEM images with high contrast between the organic surface of the microspheres and inorganic glass substrate. The original SEM image, two perpendicular profiles for two microspheres, and a histogram of the microspheres' diameters for the neat and PEGylated microspheres are illustrated in Fig. 3. The data in the histogram comply with a normal statistical distribution with the average of the microsphere diameters CH_3-$PEG_{30,000}$-NH_2-PGMA (d = 4.46 μm) > neat PGMA-COOH (d = 4.36 μm). According to this result, the PEG-coating thickness was determined as a half of the difference between diameters of PEGylated and neat particles, as approximately 50 nm.

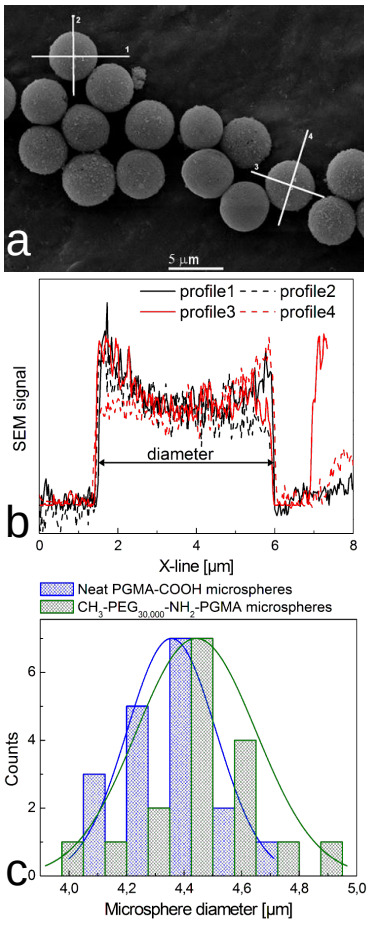

Fig. 3. (a) Scanning electron microscopy (SEM) image of CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres; (b) quantification of signal from the SEM image; and (c) comparison of diameters of neat PGMA-COOH and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres.

The Kubelka–Munk infrared spectrum of the CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres shows a clear increase in signs of the intensity of the bands around 1460 and 840 cm^{-1} in comparison to the neat PGMA-COOH microspheres. These bands (see insets in Fig. 4) correspond to the CH_2 bending and CH_2 asymmetric rocking vibrations of CH_3-$PEG_{30,000}$-NH_2 [51], respectively, and they are proof as to the presence of CH_3-$PEG_{30,000}$-NH_2 in CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres. The results of FTIR spectroscopy (Fig. 3) are in agreement with the other results confirming the PEG-coating on the microspheres. The fact that CH_3-$PEG_{30,000}$-NH_2 is chemically bound to the microspheres was confirmed by the existence of new amide bands in the FTIR spectrum of the CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres, which resulted from reaction of the terminal amine group of CH_3-$PEG_{30,000}$-NH_2 with the carboxyl group of the PGMA-COOH microspheres. This secondary amide group appears as two bands around 1670 and 1530 cm^{-1} (inset a) in Fig. 3. These bands correspond to the positions of the so-called amide I band (C = O stretching vibrations) and amide II band (combining both the N-H bending and the C-N stretching vibrations) [52].

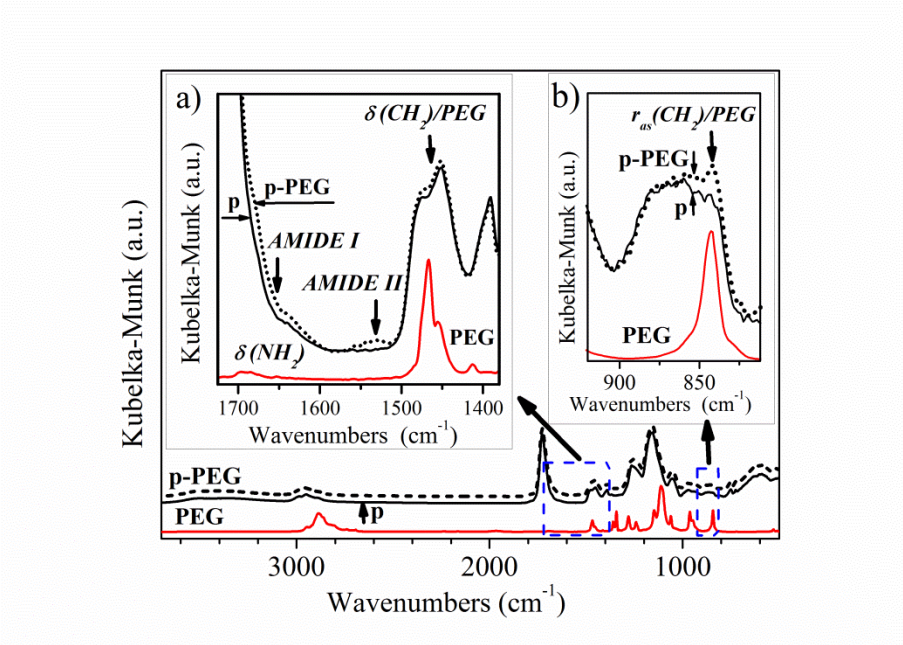

Fig. 4. The Kubelka–Munk Fourier transform infrared spectra of neat PGMA-COOH microspheres (denoted "p", solid curve), CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres ("p-PEG", dashed curve), and CH_3-$PEG_{30,000}$-NH_2 ("PEG", red curve). The insets a) and b) show the expanded areas of interest and band assignment. Refer to the text for further details and explanation.

Moreover, ELISA-like assay based on affinity interaction between biotin and streptavidin was used for monitoring of the PEG grafting on the magnetic microspheres. Particles were coated by biotin-$PEG_{2,000}$-NH_2, streptavidin-HRP conjugate was subsequently coupled, and the activity of HRP was determined. The enzyme activity corresponded to the quantity of biotinylated PEG immobilized on the microspheres. Maximum binding capacity of PGMA-COOH microspheres (1 mg) was found to be ~10 μg of biotin-

$PEG_{2,000}$-NH_2 (Fig. 5). The binding capacity of prepared PGMA-COOH microspheres was compared to those for three types of carboxyl-terminated commercial particles by this sandwich method. The absorbance, and hence the PEG density on biotin-$PEG_{2,000}$-NH_2-PGMA microspheres, was the second highest (Table 1), which is caused by the large specific surface area of the particles. Even if the assay constitutes an easy-to-handle and low-cost tool for observing the extent of PEGylation, it is nevertheless important to note that every step of the assay should be optimized. The degree of NSA of streptavidin-HRP on neat, EDC/sulfo-NHS activated, and PEGylated (biotin-free) microspheres, needs to be carefully monitored.

Table 1: List of magnetic microspheres used for PEGylation and comparison of their binding capacities as a function of HRP activity.

Microspheres	Size (μm)	Carboxyl content (mmol/g)	Absorbance (495 nm)
PGMA-COOH	4.4	0.20	1.4 ± 0.1
Dynabeads M-270 Carboxylated *	2.8	0.15	0.8 ± 0.05
Sera-Mag-Carboxyl *	0.8	0.48	1.8 ± 0.1
SiMAG-Carboxyl *	1.0	0.85	1.1 ± 0.2

* Commercially available, data from product data sheets.

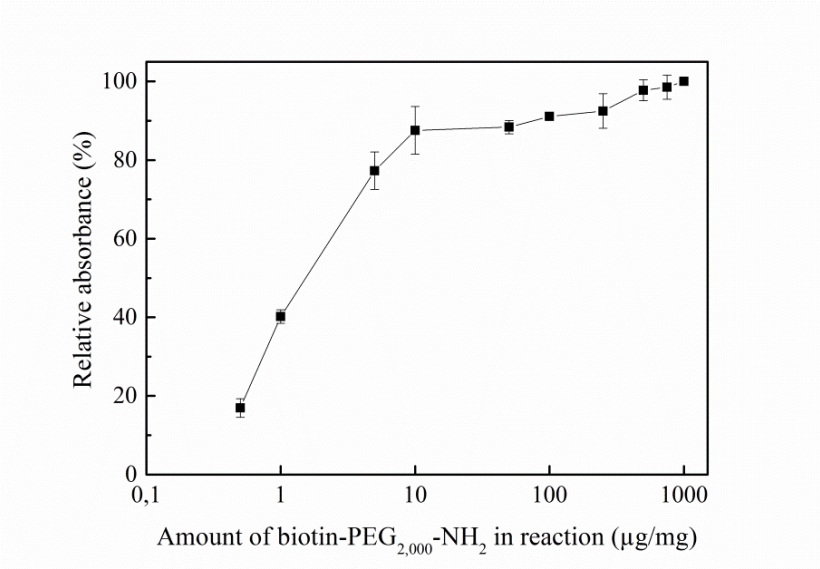

Fig. 5. Determination of maximum binding capacity of biotin-$PEG_{2,000}$-NH_2 on the surface of PGMA-COOH microspheres.

Finally, a commercial anti-PEG ELISA kit was used for specific determination of the presence of PEG on the surface of the CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres. The assay was based on competitive

ELISA, where the monoclonal anti-PEG antibodies were pre-coated onto the walls of the microtiter plate. Competitive binding between PEG on the particles and add-in PEG occurred in the next step. Therefore, the amount of signal is in this assay inversely proportional to the concentration of PEG on the microspheres. As the monoclonal anti-PEG antibodies recognized the PEG backbone with high specificity, the results from this technique clearly demonstrated that PEG chains were present on the surface of the microspheres. The absorbance of the PEGylated microspheres was comparable to that of the positive control containing 0.112 μg of PEG-BSA (Table 2).

Table 2: Qualitative proof of PEG's presence on the microspheres using competitive anti-PEG ELISA.

Microspheres	% of bound competitive PEG	SD (%)
PGMA-COOH microspheres	111	9
CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres	6	2
Negative control (no PEG)	100	-
Positive control (0.112 μg PEG-BSA)	5.6	0.3

3.3. Evaluation of behavior of PEGylated microspheres and nonspecific adsorption

3.3.1.NSA of proteins

Decreased NSA of proteins on PEGylated versus neat surfaces has been reported in several reports (see, e.g., references [35, 53-56]). An antifouling effect of PEG was observed also in this study, where the NSA of BSA on neat PGMA-COOH, CH_3-$PEG_{30,000}$-NH_2-PGMA, and CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres was compared (Fig. 6). There was no significant difference in BSA repellence between CH_3-$PEG_{30,000}$-NH_2-PGMA and CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres, and the PEG corona around the microspheres dramatically decreased NSA in both cases.

3.3.2.NSA of cells

Both neat and PEGylated microspheres were incubated with MCF7 cells as a model human cell line and NSA of cells was observed. With neat PGMA-COOH microspheres, 27 ± 2 cells were nonspecifically captured. The amount of adhered cells decreased by 51.9% in CH_3-$PEG_{2,000}$-NH_2-PGMA particles, and in CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres the decrease in NSA was even greater (74.1%) compared with the neat particles. Increased resistance against NSA of cells on PEGylated particles has been commonly exploited in drug development, where PEG hinders recognition by the body's immune system [57-59]. In our study, improved antifouling properties of magnetic microspheres due to PEGylation enabled their prospective use as a carrier for magnetic separation from complex biological material.

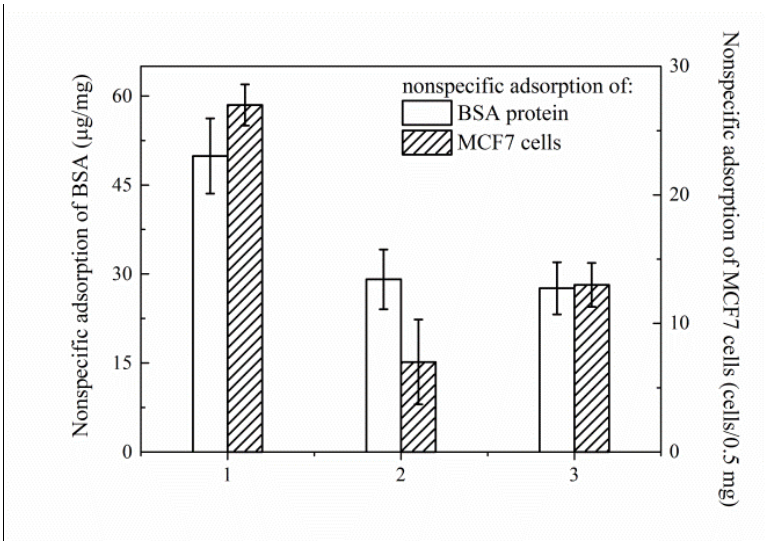

Fig. 6. Nonspecific adsorption of bovine serum albumin (BSA) and MCF7 cells on (1) neat PGMA-COOH, (2) CH_3-$PEG_{30,000}$-NH_2-PGMA, and (3) CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres.

3.3.3.Adhesion of microspheres on chip

Minimum or no adhesion together with no aggregation of particles in the channels of the chips is a key prerequisite for magnetic beads-based microfluidic bioassays. The adhesion of micrometers-sized particles on planar surfaces is a complex process and has been severally studied [60-62]. It was demonstrated, that PEGylation of magnetic particles (with a diameter of 3 μm) significantly reduced their adhesion on a glass slide [60]. In this study, two types of polymer chips – PDMS and COC - were used to compare the behavior of microspheres before and after the PEGylation. Both materials belong among the most common polymers used for biological applications of microfluidic devices [63]. PDMS is a biocompatible and nontoxic silicone elastomer permeable to gases [64]. The bottleneck of using PDMS consists in its intrinsic hydrophobicity; a lot of effort was thus spent to make its surface more hydrophilic[10, 65]. Herein, the hydrophobic nature of PDMS was retained, but the surface of the channels was first treated with a copolymer of dimethylacrylamide and allyl glycidyl ether and then passivated with BSA to reduce the nonspecific interactions with particles. The behavior of neat and PEGylated microspheres in the chip was then evaluated. The number of particles injected into each channel was $1.35 \pm 0.20 \times 10^7$ (as counted in a Bürker's chamber). After the injection, neat PGMA-COOH particles strongly aggregated and adhered to the connecting tubes and walls of the microchannel. After the rinsing and cleaning step, through which most microspheres were washed away, ten large aggregates (consisting of ca. 600 particles) remained adhered on the walls of the channels. In contrast, the adhesion of CH_3-$PEG_{2,000}$-NH_2-PGMA and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres was substantially reduced. Surprisingly, no difference in adhesion was found between particles coated with $PEG_{2,000}$ and $PEG_{30,000}$. At the end of both experiments, there was only one aggregate of PEGylated particles (containing ca. 50 particles) present in both channels, which

could have been caused by increased local surface roughness or the presence of an impurity. The second chip used for testing of adhesion of particles was made from the thermoplastic COC polymer. It is also considered as a suitable material for microfluidics [63, 66], but similarly to aforementioned PDMS, its hydrophobic interactions with biomolecules complicate the analysis of complex biological material. For this study, we used a commercial COC chip with hydrophilized channels, which were then passivated with BSA. Ideally, after introduction of particles into this chip, they should homogenously spread along the whole chamber volume, since there is a moving magnet which should assure them permanent mixing. As a result, the particles should occupy the entire volume of the chamber. After filling the channel with neat PGMA-COOH particles (1 mg), strong aggregation occurred; this was followed by the entire coverage of the walls of the chip and compact plug formation. Therefore, the manual quantification of adhered particles (as presented in the previous experiment with the PDMS chip) was not possible. In order to quantify this phenomenon (aggregation together with the adhesion of particles resulting in decreased plug size), the volumes of the particles plug in the chamber of the chip were calculated (Table 3). The neat PGMA-COOH particles occupied the smallest volume from the tested particles. In contrast, after injection of both PEGylated particles (CH_3-$PEG_{2,000}$-NH_2-PGMA and CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres), the particle adhesion was substantially reduced, which was accompanied by better spreading of the particles in the chip chamber and larger plug sizes. The results indicate that coating of particles with CH_3-$PEG_{30,000}$-NH_2 lead to higher colloidal stability and less adhesion on COC chip compared to the particles with shorter PEG (CH_3-$PEG_{2,000}$-NH_2). This is in contrast with the results of Upadhyayula et al. [60], where the effect of increasing PEG length (from 5 to 20 kDa) on increasing number of adhered particles was observed. To summarize, in both tested chips, the adhesion of PEGylated microspheres was considerably reduced compared with the neat particles. Based on these observations, it may therefore be concluded that PEGylation of PGMA-COOH microspheres improved their surface properties and behavior in the chip. Such modified microparticles are strongly suitable for microfluidic-based bioassays.

Table 3: Volumes of the plugs of the microspheres in the microfluidic chip from COC.

Microspheres	Volume of the plug ± SD (μL)
PGMA-COOH microspheres	36.3 ± 0.9
CH_3-$PEG_{2,000}$-NH_2-PGMA microspheres	53.7 ± 0.6
CH_3-$PEG_{30,000}$-NH_2-PGMA microspheres	70.0 ± 5.6

3.4. Immobilization of horseradish peroxidase as a model enzyme on PEGylated microspheres

Application of PEGylated magnetic particles in microfluidic systems is also dependent on the possibility to attach biologically active molecules, such as antibodies or enzymes. Covalent binding through PEG can result in enhanced steric accessibility of bioligand [55, 67], but, on the other hand, it can

also reduce grafting density compared to immobilization on neat surface [68]. In this study, the model enzyme (HRP) was immobilized on the microspheres modified by heterobifunctional HO-$PEG_{3,400}$-NH_2. After immobilization, the PGMA-$PEG_{3,400}$-HRP microspheres remained individual (i.e., they did not aggregate). The average enzyme activity of immobilized HRP determined from three repetitions was 0.75 ± 0.12 U . mg^{-1} of microspheres. By this biofunctionalization, the possibility for coupling of the ligand of interest was confirmed. Such carrier can be applied for in-chip catalysis.

4. Conclusion

This research team is focused on the development and biofunctionalization of magnetic micro- or nanocarriers suitable for biocatalysis or bioaffinity chromatography, often in combination with a microfluidic arrangement. After loading of the particles into a PDMS microchannel, adhesion is frequently observed, which, together with NSA of proteins, leads to failure of the experiment. Consequently, a rapid and simple method to improve particle surface properties is required. PEG was chosen for its antifouling properties, which have been so far deeply investigated in a relation with proteins, cells, DNA [53-55, 58] etc. As we know, the effect of PEGylation of magnetic microparticles on their behavior in the channels of microfluidic chips made from PDMS or COC has not been tested yet. Here, magnetic polymer particles were first PEGylated and methods for their characterization were investigated, such as zeta potential measurement, infrared spectroscopy, and SEM accompanied by image analysis. Biological assays based on affinity interactions with biotin and streptavidin proved to be reliable tools for monitoring the extent of PEGylation. A commercial anti-PEG ELISA microtiter assay was used for extremely specific determination of PEG. As a result of PEG-coating, decreased NSA of proteins and cells was observed in a batch arrangement. Then, behavior of neat and PEGylated microspheres was subsequently assessed in microfluidic PDMS devices, where substantial differences in aggregation and adhesion were recorded. Surface modification of PGMA-COOH particles with CH_3-$PEG_{30,000}$-NH_2 assured them higher repellence to the model protein BSA, cells and COC chip compared to CH_3-$PEG_{2,000}$-NH_2. In our study, PEG was used both as a tool to reduce NSA and also as a spacer for subsequent immobilization of a biologically active ligand (HRP). A universal PEG-coating procedure for magnetic microspheres together with characterization methods was described. Particles with such coating can be coupled with a ligand of interest and widely applied in microfluidics.

Acknowledgements

This study received funding from the European Community's 7th FP (2007–2013) under grant agreements no. 228980, CAMINEMS, no. 246513, NADINE, no. 317742, LOVE-FOOD.

References:

1 N. T. Thanh, Magnetic Nanoparticles: From Fabrication to Clinical Applications, 1st ed., CRC Press, Boca Raton, 2012.

2 S. C. McBain, H. H. P. Yiu and J. Dobson, Int. J. Nanomed., 3 (2008), 169. (DOI: http://dx.doi.org/10.2147/IJN.S1608)

3 L. Zhang, F. X. Gu, J. M. Chan, A. Z. Wang, R. S. Langer and O. C. Farokhzad, Clin. Pharmacol. Ther., 83 (2008), 761. (DOI:10.1038/sj.clpt.6100400).

4 I. Safarik and M. Safarikova, Chem. Pap., 63 (2009), 497 (DOI:10.2478/s11696-009-0054-2).

5 M. A. M. Gijs, F. Lacharme and U. Lehmann, Chem. Rev., 110 (2009), 1518 (DOI: 10.1021/cr9001929)

6 J. Choi, K. Oh, J. Thomas, W. Heineman, H. Halsall, J. Nevin, A. Helmicki, H. Henderson and C. Ahn, Lab Chip, 2 (2002), 27. (DOI:10.1039/b1077540n).

7 M. Slovakova, N. Minc, Z. Bilkova, C. Smadja, W. Faigle, C. Futterer, M. Taverna and J. Viovy, Lab Chip, 5 (2005), 935 (DOI:10.1039/b504861c).

8 Z. Fan, S. Mangru, R. Granzow, P. Heaney, W. Ho, Q. Dong and R. Kumar, Anal. Chem., 71 (1999), 4851. (DOI:10.1021/ac9902190).

9 M. Hayes, N. Polson, A. Phayre and A. Garcia, Anal. Chem., 73 (2001), 5896. (DOI:10.1021/ac0104680).

10 D. Wu, B. Zhao, Z. Dai, J. Qin and B. Lin, Lab Chip, 6 (2006), 942. (DOI:10.1039/b600765a).

11 L. Roach, H. Song and R. Ismagilov, Anal. Chem., 77 (2005), 785. (DOI:10.1021/ac049061w).

12 M. Poitevin, Y. Shakalisava, S. Miserere, G. Peltre, J. Viovy and S. Descroix, Electrophoresis, 30 (2009), 4256. (DOI:10.1002/elps.200900254).

13 A. Pallandre, B. de Lambert, R. Attia, A. Jonas and J. Viovy, Electrophoresis, 27 (2006), 584. (DOI:10.1002/elps.200500761).

14 V. Audonnet, L. Malaquin and J. Viovy, Bioanal. Rev., 3 (2011), 41. (DOI:10.1007/s12566-011-0022-5).

15 U. Wattendorf and H. P. Merkle, J. Pharm. Sci., 97 (2008), 4655. (DOI:10.1002/jps.21350).

16 J. M. Goddard and J. H. Hotchkiss, Prog. Polym. Sci., 32 (2007), 698. (DOI:10.1016/j.progpolymsci.2007.04.002).

17 P. Gong and D. W. Grainger, Nonfouling Surfaces, in J. B. Rampal (Ed.), Methods in Molecular Biology, vol. 381: Microarrays, 2nd Edition: Vol. 1: Synthesis Methods, 2nd ed., Humana Press, Totowa, New Jersey, 2007, pp. 59-92.

18 C. Fee and J. Van Alstine, Chem. Eng. Sci., 61 (2006), 924. (DOI:10.1016/j.ces.2005.04.040).

19 S. Jevsevar, M. Kunstelj and V. G. Porekar, Biotechnol. J., 5 (2010), 113. (DOI:10.1002/biot.200900218).

20 H. Hlídková, D. Horák, V. Proks, Z. Kučerová, M. Pekárek and J. Kučka, Macromol. Biosci., 13 (2013), 503. (DOI:10.1002/mabi.201200446).

21 R. Fields, Biochem. J., 124 (1971), 581.

22 S. Udenfriend, S. Stein, P. Böhlen and W. Dairman, Science, 178 (1972), 871. (DOI:10.1126/science.178.4063.871).

23 B. Kendrick, B. Kerwin, B. Chang and J. Philo, Anal. Biochem., 299 (2001), 136. (DOI:10.1006/abio.2001.5411).

24 J. Bullock, S. Chowdhury, A. Severdia, J. Sweeney, D. Johnston and L. Pachla, Anal. Biochem., 254 (1997), 254. (DOI: 10.1006/abio.1997.2405).

25 P. Yu, G. Qin, D. Qin and Z. Zhang, Anal. Biochem., 396 (2010), 325. (DOI: 10.1016/j.ab.2009.10.006).

26 T. Yu, J. A. Traina, E. Pungor Jr. and M. McCaman, Anal. Biochem., 359 (2006), 54. (DOI:10.1016/j.ab.2006.08.018).

27 B. K. Seyfried, J. Siekmann, O. Belgacem, R. J. Wenzel, P. L. Turecek and G. Allmaier, J. Mass Spectrom., 45 (2010), 612. (DOI:10.1002/jms.1746).

28 S. Chowdhury, M. Doleman and D. Johnston, J. Am. Soc. Mass Spectrom., 6 (1995), 478. (DOI:10.1016/1044-0305(95)00190-O).

29 D. Na, E. Park, Y. Youn, B. Moon, Y. Jo, S. Lee, W. Kim, Y. Sohn and K. Lee, Electrophoresis, 25 (2004), 476. (DOI:10.1002/elps.200305684).

30 X. Li, Q. Yang, J. Yan, K. Xu, J. Liu, X. He, X. Zhang and Q. Liu, Artif. Cells Blood Substit. Biotechnol., 35 (2007), 1. (DOI:10.1080/10731190600974210).

31 X. W. Gong, D. Z. Wei, M. L. He and Y. C. Xiong, Talanta, 71 (2007), 381. (DOI:10.1016/j.talanta.2006.04.010).

32 M. Kurfurst, Anal. Biochem., 200 (1992), 244. (DOI:10.1016/0003-2697(92)90460-O).

33 M. Garcia-Fuentes, D. Torres, M. Martin-Pastor and M. Alonso, Langmuir, 20 (2004), 8839. (DOI:10.1021/la049505j).

34 J. Dust, Z. Fanf and J. Harris, Macromolecules, 23 (1990), 3742. (DOI:10.1021/ma00218a005).

35 E. K. U. Larsen and N. B. Larsen, Lab Chip, 13 (2013), 669. (DOI:10.1039/c2lc40750g).

36 D. Horák, J. Kučerová, L. Korecká, B. Jankovičová, J. Palarčík, P. Mikulášek and Z. Bílková, Macromol. Biosci., 12 (2012), 647. (DOI:10.1002/mabi.201100393).

37 J. Ugelstad, T. Ellingsen, A. Berge, B. Helgee, WO 83/03920 (1983).

38 J. Staros and B. Kakkad, J. Membr. Biol., 74 (1983), 247. (DOI:10.1007/BF02332127).

39 P. Sahoo, S. Soltani, A. Wong and Y. Chen, Computer Vision Graphics and Image Processing, 41 (1988), 233. (DOI:10.1016/0734-189X(88)90022-9).

40 P. Knotek, M. Vlcek, M. Kincl and L. Tichy, Thin Solid Films, 520 (2012), 5472. (DOI:10.1016/j.tsf.2012.03.116).

41 P. Klapetek, D. Nečas and C. Anderson, Gwyddion - Free SPM data analysis software; v.2.25., (2011).

42 P. Kubelka and F. Munk, Z. Tech. Phys., 12 (1931), 593.

43 J. Bovaird, T. Ngo and H. Lenhoff, Clin. Chem., 28 (1982), 2423.

44 Y. Xia and G. Whitesides, Annu. Rev. Mater. Sci., 28 (1998), 153. (DOI:10.1146/annurev.matsci.28.1.153).

45 L. Saias, J. Autebert, L. Malaquin and J. Viovy, Lab Chip, 11 (2011), 822. (DOI:10.1039/c0lc00304b).

46 M. Chiari, M. Cretich, F. Damin, L. Ceriotti and R. Consonni, Electrophoresis, 21 (2000), 909. (DOI:10.1002/(SICI)1522-2683(20000301)21:5<909::AID-ELPS909>3.0.CO;2-L).

47 J. Turkova, J. Vajcner, D. Vancurova and J. Stamberg, Collect. Czech. Chem. Commun., 44 (1979), 3411.

48 G. T. Hermanson, Bioconjugate Techniques 2nd Edition., Academic Press, Amsterdam, 2008.

49 R. Gref, M. Lűck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk and R. H. Műller, Colloid Surf. B-Biointerfaces, 18 (2000), 301. (DOI: 10.1016/S0927-7765(99)00156-3).

50 Y. Li, Y. Pei, X. Zhang, Z. Gu, Z. Zhou, W. Yuan, J. Zhou, J. Zhu and X. Gao, J. Control. Release, 71 (2001), 203. (DOI: 10.1016/S0168-3659(01)00218-8).

51 K. A. Rubinson and C. W. Meuse, Polymer, 54 (2013), 709. (DOI:10.1016/j.polymer.2012.11.016).

52 G. Socrates, Infrared characteristic group frequencies: Tables and charts, 2nd Edition, Wiley, New York, 1997.

53 M. Muller, J. Voros, G. Csucs, E. Walter, G. Danuser, H. Merkle, N. Spencer and M. Textor, J. Biomed. Mater. Res. Part A, 66A (2003), 55. (DOI:10.1002/jbm.a.10502).

54 J. Bluemmel, N. Perschmann, D. Aydin, J. Drinjakovic, T. Surrey, M. Lopez-Garcia, H. Kessler and J. P. Spatz, Biomaterials, 28 (2007), 4739. (DOI:10.1016/j.biomaterials.2007.07.038).

55 H. Chen, Z. Zhang, Y. Chen, M. A. Brook and H. Sheardown, Biomaterials, 26 (2005), 2391. (DOI: 10.1016/j.biomaterials.2004.07.068).

56 Y. Nagasaki, H. Kobayashi, Y. Katsuyama, T. Jomura and T. Sakura, J. Colloid Interface Sci., 309 (2007), 524. (DOI:10.1016/j.jcis.2006.12.079).

57 J. Xie, C. Xu, N. Kohler, Y. Hou and S. Sun, Adv. Mater., 19 (2007), 3163. (DOI:10.1002/adma.200701975).

58 T. Betancourt, J. D. Byrne, N. Sunaryo, S. W. Crowder, M. Kadapakkam, S. Patel, S. Casciato and L. Brannon-Peppas, J. Biomed. Mater. Res. Part A, 91A (2009), 263. (DOI:10.1002/jbm.a.32247).

59 O. Kinstler, G. Molineux, M. Treuheit, D. Ladd and C. Gegg, Adv. Drug Deliv. Rev., 54 (2002), 477. (DOI: 10.1016/S0169-409X(02)00023-6).

60 S. Upadhyayula, T. Quinata, S. Bishop, S. Gupta, N. R. Johnson, B. Bahmani, K. Bozhilov, J. Stubbs, P. Jreij, P. Nallagatla and V. I. Vullev, Langmuir, (28) 2012, 5059. (DOI:10.1021/la300545v).

61 K. Perez-Toralla, J. Champ, M. R. Mohamadi, O. Braun, L. Malaquin, J. Viovy and S. Descroix, Lab Chip, 13 (2013), 4409. (DOI:10.1039/c3lc50888a).

62 B. Teste, F. Malloggi, J. Siaugue, A. Varenne, F. Kanoufi and S. Descroix, Lab Chip, 11 (2011), 4207. (DOI:10.1039/c1lc20809h).

63 A. Alrifaiy, O. A. Lindahl and K. Ramser, Polymers, 4 (2012), 1349. (DOI:10.3390/polym4031349).

64 A. Mata, A. Fleischman and S. Roy, Biomed. Microdevices, 7 (2005), 281. (DOI:10.1007/s10544-005-6070-2).

65 J. Zhou, D. A. Khodakov, A. V. Ellis and N. H. Voelcker, Electrophoresis, 33 (2012), 89. (DOI:10.1002/elps.201100482).

66 H. Becker and C. Gartner, Anal Bioanal Chem. 390 (1) (2008), 89. (DOI: 10.1007/s00216-007-1692-2).

67 C. Manta, N. Ferraz, L. Betancor, G. Antunes, F. Batista-Viera, J. Carlsson and K. Caldwell, Enzyme Microb. Technol., 33 (2003), 890. (DOI: 10.1016/S0141-0229(03)00221-7).

68 B. Thierry, M. Kurkuri, J. Y. Shi, E. M. P. Lwin and D. Palms, Biomicrofluidics, 4 (2010), 32205. (DOI:10.1063/1.3480573).