European Polymer Journal 47 (2011) 1090–1096

Contents lists available at ScienceDirect

European Polymer Journal

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

Streptavidin-modified magnetic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) microspheres for selective isolation of bacterial DNA

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ARTICLE INFO

Article history:
Received 13 November 2010
Received in revised form 1 February 2011
Accepted 10 February 2011
Available online 16 February 2011

Keywords:
Glycidyl methacrylate
2-hydroxyethyl methacrylate
Magnetic
Microspheres
DNA

ABSTRACT

The dispersion copolymerization of 2-hydroxyethyl methacrylate (HEMA) with glycidyl methacrylate (GMA) in a toluene/2-methylpropan-1-ol mixture in the presence of Fe_3O_4 nanoparticles coated with oleic acid, produced monodisperse magnetic poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) (P(HEMA-GMA)) microspheres. Oxirane groups of the microspheres were ammonolyzed and then functionalized with streptavidin using cyanuric chloride. The final product contained 0.67 mg of streptavidin per gram of wet magnetic P(HEMA-GMA) microspheres. The microspheres were characterized by elemental analysis, scanning electron microscopy, IR, UV–VIS and atomic absorption spectroscopy. The streptavidin-modified magnetic P(HEMA-GMA) microspheres were used for immobilization of biotinylated DNA and subsequent selective isolation of target DNA from complex samples using DNA/DNA hybridisation. Based on the highly selective recognition of streptavidin with a biotin-labeled DNA probe, DNA sensor was constructed for magnetic separation of DNA from real samples.

1. Introduction

Methods based on antigen–antibody interaction have been used in many fields, such as clinical diagnosis, environment monitoring and food safety [1,2]. Streptavidin, a tetrameric protein of molecular weight 60 kDa, which can bind four biotin molecules, finds wide use in in molecular biology and biotechnology. The high and specific affinity of streptavidin to biotin (association constant, $K_a \approx 10^{15}$) is well known and their coupling has been used for rapid isolation of proteins [3,4] or for preparation of biosensors [5–7]. In many reports, streptavidin coupled to magnetic beads has been widely used in various applications [8–11].

An ideal solid support for immobilization of proteins should meet several requirements. To immobilize an abundant antigen or antibody, it should be hydrophilic to avoid nonspecific interactions with analytes and the sample matrix. Magnetic carriers are becoming increasingly popular as they make it possible to overcome problems associated with polymer separation from solution. The fact that magnetic separation obviates centrifugation or filtration is one of the benefits of magnetic microspheres.

Polymerase chain reaction (PCR) has become a powerful diagnostic tool for analysis of microorganisms. The occurrence of falsely negative results is a problem in routine testing of real samples using PCR. Falsely negative results can be caused by the presence of intra- or extracellular inhibitors [12]. For this reason, the optimal extraction method for obtaining DNA in PCR-ready quality is crucial for detection of target cells in real samples. The problem can be solved by various isolation and purification methods. Therefore, the adsorption of total DNA using non-porous magnetic carboxyl-functionalized poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate) (P(HEMA-GMA)) microspheres in the presence of high concentrations of poly(ethylene glycol) (PEG 6000) and sodium chloride was developed. This method has been

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doi:10.1016/j.eurpolymj.2011.02.007

successfully used for isolation of the total DNA from samples containing PCR inhibitors [13,14].

In this communication, preparation of magnetic P(HEMA-GMA) microspheres with attached streptavidin is described. While the presence of HEMA units provides hydrophilicity of the particles, the reactive oxirane groups are suited for subsequent immobilization of streptavidin. The aim of this work was to investigate selective isolation of bacterial DNA using magnetic P(HEMA-GMA) microspheres functionalized with streptavidin and biotinylated DNA probe.

2. Experimental

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA; both from Röhm GmbH, Germany) were vacuum-distilled before use. Dibenzoyl peroxide (BPO) was purchased from Fluka (Buchs, Switzerland) and used without further purification. Cellulose acetate butyrate (CAB; M_n = 100,000; 35/15 acetyl/butyryl; Eastman, Kingsport, USA) was a stabilizer. Ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS) and all other chemicals were supplied by Sigma (St. Louis, USA) and used as received. The buffer for immobilization of streptavidin was 0.01 M phosphate buffer (PBS; pH 7.4). All solutions were prepared from ultrapure Q water ultrafiltered on a Milli-Q Gradient A10 system (Millipore, Molsheim, France). The PCR and biotinylated primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic). Taq polymerase was from Top-Bio (Prague, Czech Republic).

2.2. Precipitation of magnetic iron oxide

Precipitation of magnetite and its coating with oleic acid was described previously [15,16]. Briefly, $FeCl_3 \cdot 6H_2O$ (24.32 g) and 11.92 g of $FeCl_2 \cdot 4H_2O$ (mole ratio 2:1) were stirred in 50 mL of double-distilled water under nitrogen. After equilibration, 50 mL (50% excess) of a 28% NH_3 solution was added to the mixture over a period of 20–30 min. To the reaction mixture at 90 °C, 5 mL of oleic acid was added. The reaction proceeded for 5 h until NH_3 disappeared. After cooling to room temperature, the nanoparticles were washed with water for 4 days (3 × 200 mL a day). The magnetite particles were immobilized with a magnet below the flask bottom, decanted, and washed with water. After drying at 80 °C and 13 Pa, about 16 g of nanoparticles was obtained.

2.3. Preparation of magnetic P(HEMA-GMA) microspheres

Magnetic P(HEMA-GMA) microspheres were prepared by dispersion polymerization in a 100-ml flask equipped with a mechanical stirrer [15]. Toluene (38.2 g) was added to Fe_3O_4 (1 g) coated with oleic acid and the mixture was sonicated for 20 min with a W-385 ultrasonic homogenizer (Heat Systems-Ultrasonics, Farmingdale, USA) at 50% power input. Spontaneous redispersion occurred, forming a homogeneous black dispersion. Then, a solution of CAB (3.2 g) in 2-methylpropan-1-ol (29.8 g) was added and, after sonication, the flask containing the dispersion was mounted to a reactor. HEMA (6 g), GMA (6 g) and BPO (0.24 g) were added, and the solution was deoxygenated by purging with nitrogen for 10 min. The reaction was run at 70 °C with stirring (500 rpm) for 17 h. The resulting particles were purified by repeated magnetic separation from a toluene/2-methylpropan-1-ol mixture with the gradually decreasing alcohol content and decantation until excess CAB was removed and a solid in the supernatant disappeared. Then, the product from toluene was dried.

2.4. Reaction of magnetic P(HEMA-GMA) microspheres with ammonia solution

Magnetic P(HEMA-GMA) microspheres (3 g) were mixed with 19% aqueous ammonia solution (20 mL) and stirred at 23 °C for 24 h. After the reaction, the P(HEMA-GMA)-NH_2 microspheres were washed twice with water (20 mL).

2.5. Streptavidin functionalization of magnetic P(HEMA-GMA)-NH_2 microspheres

A solution of cyanuric chloride (3 mg) in dioxane (1 mL) was added to a dispersion of P(HEMA-GMA)-NH_2 microspheres (2.2 g) in a dioxane/water (3/2 v/v) solution (5 mL) under stirring; the reaction proceeded at 0 °C for 3 min. The microspheres were washed with ice-cold dioxane/water (3/2 v/v) solution, then dispersed in a solution of streptavidin (5 mg) in PBS (5 mL; pH 7.4), 0.1 M Na_2HPO_4 (2 mL) was added and the mixture was stirred at laboratory temperature for 45 min. The resulting P(HEMA-GMA)-NH_2-STV microspheres were separated magnetically and a UV spectrum of the solution was measured. To retain the activity of the bonded streptavidin and to suppress bacterial fouling, a 0.1% BSA (bovine serum albumin) solution in PBS buffer and 0.02% aqueous sodium azide solution were added to the suspension.

2.6. Magnetic P(HEMA-GMA)-NH_2-STV microspheres with biotinylated DNA probe

Biotinylated primer 5′bioRpaca from primer pair specific for *Lactobacillus paracasei* [17] was immobilized on magnetic P(HEMA-GMA)-NH_2-STV microspheres by modification of a previously described procedure [18]. Biotinylated primer (500 pmol, 25 μL) was mixed with 1 M NaCl solution (25 μL), the mixture (50 μL) was added to a suspension of magnetic P(HEMA-GMA)-NH_2-STV microspheres in 2 M KCl solution (50 μL) and incubated at laboratory temperature for 5 min. The magnetic P(HEMA-GMA)-NH_2-STV microspheres with immobilized biotinylated DNA probe were washed twice with 2 M NaCl solution (100 μL each).

2.7. Cell cultivation and DNA isolation

The *L. paracasei* CCDM 211 strain was obtained from the Culture Collection of Dairy Microorganisms (CCDM, Tábor,

Czech Republic). The bacteria were aerobically grown in the Man, Rogosa, Sharp (MRS) medium (Oxoid, London, UK) at pH 6.5–7.0 and 37 °C for 24 h. The bacteria were resuspended in 500 μL of lysis buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.8) supplemented with lysozyme (3 mg/mL) and incubated at 20 °C for 1 h. 10 μL of proteinase K (100 μg/mL) and a 20% aqueous SDS solution (12.5 μL) were added to each sample and the mixtures were incubated at 55 °C overnight. The crude cell lysates were used in DNA extraction. Commercial BIFI-Pangamin probiotic pills (food supplement containing defatted milk) served as a complex matrix. The pills (1 g) were pulverized and resuspended in 5 mL of lysis buffer with lysozyme (see above). The cell lysis and DNA isolation were performed as above. The integrity of nucleic acids was confirmed by gel electrophoresis and the DNA purity was determined by UV spectrophotometry using the $A_{260\ nm}/A_{280\ nm}$ ratio [19]. The immobilized DNA probe was used for isolation of the denatured *L. paracasei* DNA (*via* DNA/DNA hybridization). Denaturated DNA (25 μL, 99 °C for 2 min in TE buffer) was mixed with 1 M NaCl solution (25 μL). The mixture (50 μL) was added to a suspension of magnetic P(HEMA-GMA)-NH_2-STV microspheres with immobilized probe in 1 M NaCl solution (50 μL) and incubated at laboratory temperature for 5 min. The complex (functionalized microspheres – DNA) was repeatedly washed with 2 M NaCl solution and separated by a magnetic separator (Invitrogen Dynal AS, Oslo, Norway). Hybridized DNA was recovered by heating (70 °C for 4 min) in TE buffer (50 μL). Finally, the microspheres were magnetically separated and DNA eluate was transferred in a new tube. DNA extracted with phenol and precipitated in ethanol served as a control [20].

2.8. PCR amplification and agarose gel electrophoresis

DNA isolated on the tested magnetic microspheres was amplified in PCR with the *Lactobacillus* genus-specific primers LbLMA 1-rev and R16–1 [21]. The resulting DNA amplicons had approximately 250 bp length. PCR mixture contained 10 mM dNTP (0.5 μL), 0.5 μL of each primer (10 pmol/μL), 1 μL of Taq 1.1 polymerase (1 U/μL), PCR buffer (2.5 μL) with 1.5 mM Mg^{2+} and 1 μL of DNA matrix (10 ng/μL); PCR water was added up to the 25 μL volume. The amplification reactions were carried out using the following cycle parameters: 5 min of the initial denaturation period at 95 °C (hot start), 30 s of denaturation at 95 °C, 30 s of primer annealing at 55 °C, and 30 s of extension at 72 °C. The final polymerization step was prolonged to 10 min; the number of cycles was 35. The PCR products (250 bp) were detected by agarose gel electrophoresis (1.8%).

2.9. Characterization

To observe Fe_3O_4 nanoparticles, their dispersion in toluene was diluted and sprayed on a grid with a carbon membrane and viewed with a JEOL JEM 200 CX transmission electron microscope (Tokyo, Japan). Morphology of the magnetic P(HEMA-GMA) microspheres was characterized with a Vega Plus TS 5135 scanning electron microscope (Tescan Brno, Czech Republic). A Perkin-Elmer Lambda 20 UV/VIS spectrophotometer (Norwalk, CT, USA) was used for UV measurements. IR spectra of the microspheres were recorded on a Paragon 1000 PC Perkin-Elmer FT-IR spectrometer and the oxirane group content was determined [22] by analysis of the peak at 910 cm^{-1}. The amount of iron in the microspheres was analyzed with a Perkin-Elmer 3110 atomic absorption spectrometer (AAS). A microsphere extract was obtained with dilute HCl (1:1) at 80 °C for 1 h. Elemental analyses were performed using a Perkin-Elmer model 2400 CHNS/O analyzer.

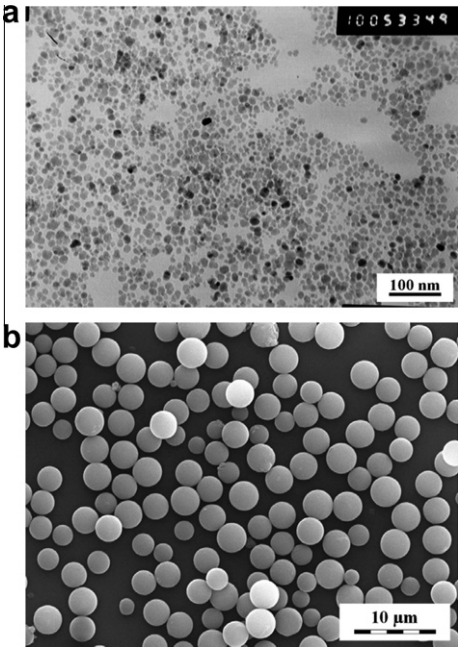


Fig. 1. (a) TEM micrograph of Fe_3O_4 nanoparticles coated with oleic acid and (b) SEM micrograph of magnetic P(HEMA-GMA) microspheres.

3. Results and discussion

3.1. Magnetic nanoparticles

Superparamagnetic magnetite (Fe_3O_4) nanoparticles were prepared by chemical co-precipitation of Fe(II) and Fe(III) salts with an aqueous NH_3 solution at 90 °C. Fe_3O_4 nanoparticle dispersions in water were stable due to electrostatic stabilization with ammonium ion double layers. The added oleic acid was adsorbed on the surface of magnetite particles due to the complexation of carboxyl groups with iron ions. First, a magnetic fluid concentrate was prepared which was washed many times with distilled water to remove all contaminants. Transfer of magnetite particles from aqueous to toluene medium was performed using peptization which yielded an asphalt-like paste from which water was easily removed. The paste free of water was then dispersed in toluene by ultrasound to give ferrofluid. A typical TEM image of nanoparticles coated with oleic acid in toluene medium (Fig. 1a) indicated their diameter range 10 ± 5 nm. Good dispersibility of the coated

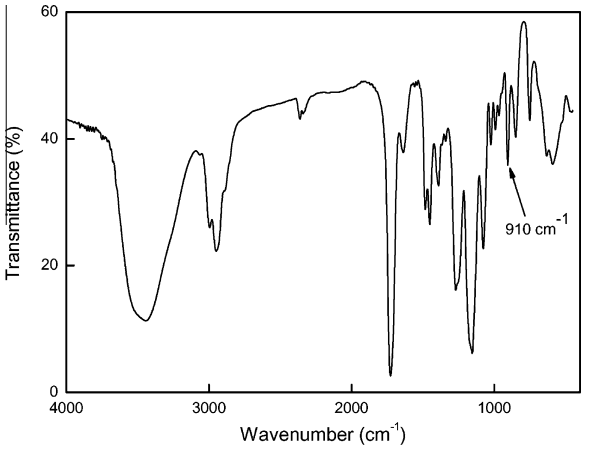
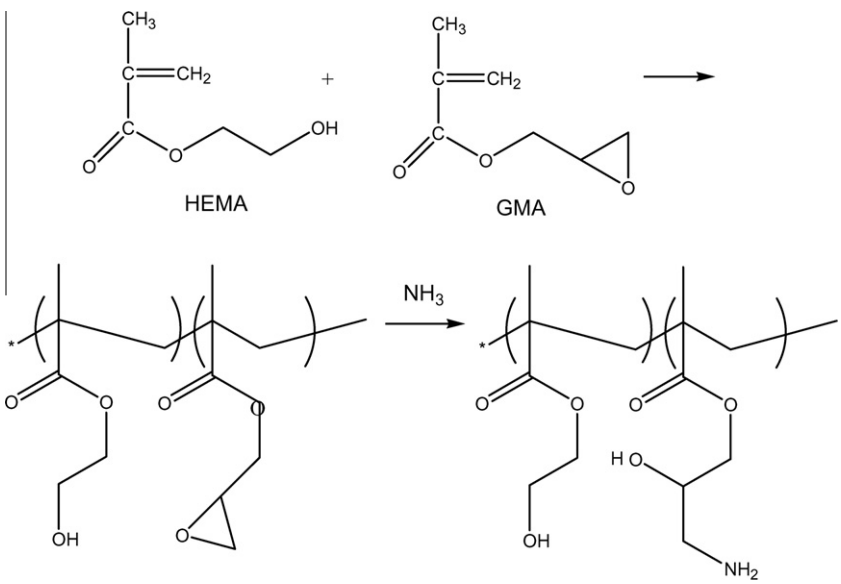


Fig. 2. IR spectrum of magnetic P(HEMA-GMA) microspheres.

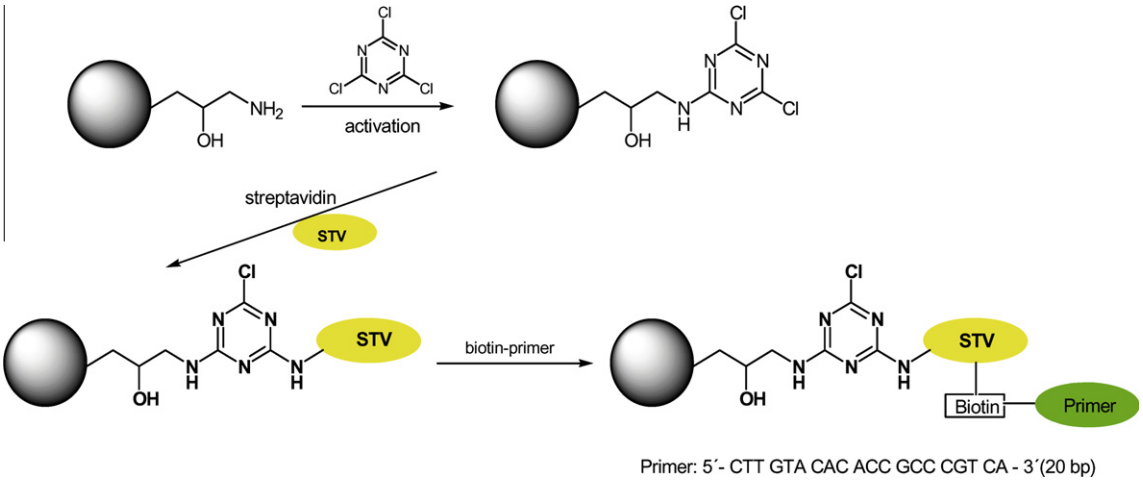
Fe_3O_4 nanoparticles in nonpolar solvents was a consequence of their hydrophobization [23]. The long alkyls of oleic acid not only stabilized the Fe_3O_4 nanoparticles preventing their coagulation but also enhanced the affinity of the nanoparticles to the monomer in the following polymerization step.

3.2. Magnetic microspheres

Magnetite nanoparticles were then encapsulated by dispersion polymerization, which is a new development in producing magnetic microspheres. Dispersion polymerization is an intermediate technique between homogeneous and heterogeneous polymerizations. It is based on the finding that while the monomers (HEMA and GMA), initiator (BPO) and stabilizer (CAB) are soluble in the polymerization medium (toluene/2-methylpropan-1-ol), the polymer is insoluble. In order to achieve incorporation of magnetite nanoparticles in the latex, they were first dispersed in the polymerization medium. The advantage of coating with oleic acid, containing carboxyl groups readily adhering to the surface of iron oxide nanoparticles, is that it provides hydrophobic environment necessary for solubilization of monomers (HEMA and GMA). As oligomer chains grow and eventually reach a molecular weight exceeding a critical value, they precipitate from solution on magnetite nanoparticles and aggregate to form unstable colloidal precursor particles (nuclei) [24]. These particles coalesce and adsorb the stabilizer from the reaction medium on their surface until they become stable colloids. The procedure, however, does not make it possible to completely avoid the formation of free polymer, which almost always competes with the coating. Nevertheless, magnetite encapsulation in polymer microspheres, in spite of adsorption of magnetic material on the particle, is advantageous because the microsphere with adsorbed magnetic nanoparticles cannot be considered a stable magnetic microsphere as the adsorbed magnetic nanoparticle may detach from the surface during washing or changing temperature or pH of the medium. Moreover, surface iron oxide could inhibit the activity of biomolecules. The dispersion copolymerization of HEMA and GMA in the presence of Fe_3O_4 nanoparticles coated with oleic acid produced magnetic P(HEMA-GMA) microspheres. They were monodisperse, 2.9 μm in diameter (Fig. 1b) as documented by the polydispersity index (weight- to number-average particle size) PDI = 1.04. The particles contained 3.5 mmol oxirane groups per gram (which corresponds to the theoretical value given by the recipe) as found by analysis of the 910 cm^{-1} peak in IR spectra (Fig. 2) and 5.94 wt.% Fe (by AAS) [15]. The microspheres were non-porous, therefore, their specific surface area was low (~1 m^2/g). The effect of a number of adjustable process parameters, such as type and concentration of the monomer, the



Scheme 1. Copolymerization of HEMA with GMA and ammonolysis of the copolymer.



Scheme 2. Functionalization of P(HEMA-GMA)-NH_2 microspheres with streptavidin and DNA probe (biotinylated primer).

stabilizer and the initiator, the temperature, the composition of the solvent system, and the amount of the ferrofluid were studied in our previous report [15]. Magnetic characteristics of P(HEMA-GMA) microspheres prepared by dispersion polymerization in the presence of ferrimagnetic nanoparticles were also described earlier [25].

A disadvantage of dry magnetic P(HEMA-GMA) microspheres consists in their poor wettability with water. Dispersing in water using surfactants that adsorb on the particle surface, thus influencing subsequent reactions with biomolecules, is undesirable if proteins are immobilized on the particles. The oxirane groups of the microspheres were therefore ammonolysed.

3.3. Immobilization of streptavidin

In order to activate magnetic P(HEMA-GMA) microspheres, their oxirane groups were transformed to amino groups by treatment with aqueous ammonia (Scheme 1). The elemental analysis of the resulting magnetic P(HEMA-GMA)-NH_2 microspheres confirmed the presence of nitrogen (4.09 wt.%) indicating that 2.6 mmol of amino groups were introduced per gram of the polymer. This roughly corresponded to 73% conversion of oxirane to amino groups. The lower amount of amino groups can be the result of internal crosslinking or inaccessibility of some oxirane groups. After ammonolysis, the microspheres became sufficiently hydrophilic and could thus be well dispersed in water. Then they were activated with cyanuric chloride and functionalized with streptavidin (Scheme 2). The conjugation of streptavidin with P(HEMA-GMA)-NH_2 was verified by monitoring UV spectra (270 nm) of the reaction mixture both before and after immobilization of streptavidin (Fig. 3). By analysis of UV spectra it was found that 0.67 mg of streptavidin was bound per gram of wet magnetic P(HEMA-GMA)-NH_2 microspheres. Such a relatively low amount of the attached streptavidin is convenient from the point of view of preventing the multilayer immobilization. It is interesting to note that the reaction was very fast taking only 3 min and its prolongation to 75 min gave the same contents.

3.4. Isolation of bacterial DNA on streptavidin-modified magnetic P(HEMA-GMA)-NH_2 microspheres

To verify the applicability of the developed magnetic P(HEMA-GMA)-NH_2-STV microspheres, DNA probe was immobilized and tested in isolation of *L. paracasei* DNA. Immobilization of biotinylated primer was performed using the previously described procedure [18]. The microspheres with immobilized DNA probe were used for separation of denatured DNA isolated from *L. paracasei*. The interaction of single-stranded molecules of probe and target DNA must show a high degree of base complementarity. To control the progress of the target DNA isolation, various amounts of DNA from *L. paracasei* were tested in hybridization (125 ng–1.25 pg).

DNA eluted from the magnetic P(HEMA-GMA)-NH_2-STV microspheres was amplified in PCR, yielding products of different intensities depending on the amount of target

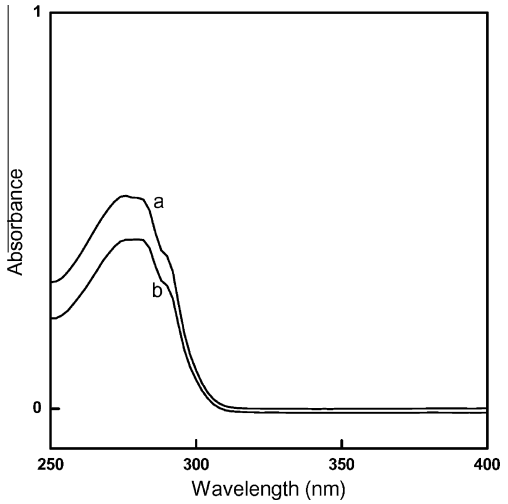


Fig. 3. UV–VIS spectra of a reaction solution (a) before and (b) after immobilization of streptavidin on magnetic P(HEMA-GMA)-NH_2 microspheres. (Measured against phosphate buffer, pH 7.4, as a reference).

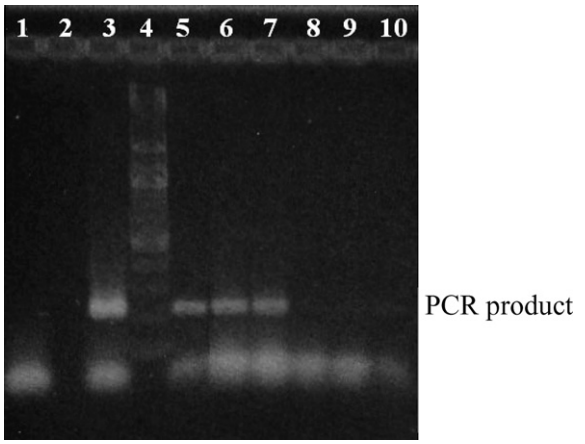


Fig. 4. Agarose gel electrophoresis of PCR products obtained by amplification of *Lactobacillus paracasei* DNA separated on magnetic P(HEMA-GMA)-NH_2-STV microspheres.Conditions: 1.5% agarose gel, TBE buffer (45 mM boric acid, 45 mM Tris-base, 1 mM EDTA, pH 8.0). Lane 1: negative control without DNA, lane 2: void, lane 3: positive control with *L. paracasei* DNA (10 ng), lane 4: DNA standard (100 bp ladder), lanes 5–10: *Lactobacillus paracasei* DNA (125, 12.5, 1.25, 0.125, 0.0125, and 0.00125 ng).

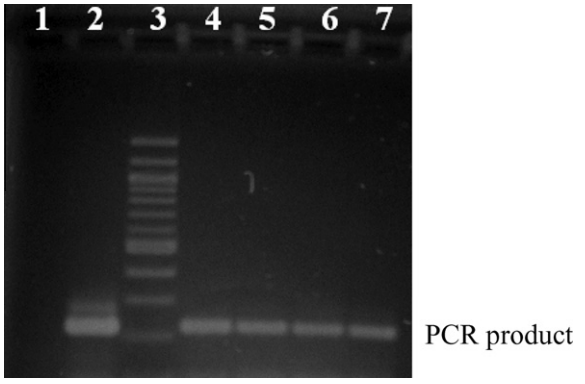


Fig. 5. Agarose gel electrophoresis of PCR products (250 bp) specific for genus *Lactobacillus*. Conditions: see Fig. 4. Lane 1: negative control, lane 2: positive control (10 ng DNA *Lactobacillus paracasei*), lane 3: DNA standard (100 bp ladder), lanes 4–7: DNA isolated from four BIFI-Pangamin pills.

DNA in eluate. Specific PCR products of high intensities were detected if 1.25 ng DNA (or more) were used for hybridization (Fig. 4). This amount of DNA corresponds approximately to 10^5 *Lactobacillus* cells. PCR products of very low intensities were detected using 125–1.25 pg DNA for hybridization. Finally, *Lactobacillus* DNA was separated from the total DNA isolated from a probiotic food supplement (BIFI-Pangamin pills) on P(HEMA-GMA)-NH_2-STV microspheres functionalized with biotinylated primer. According to the manufacturer, the pills contain *Lactobacillus* and *Bifidobacterium* cells and brewer's yeast *Saccharomyces cerevisiae*. The results of amplification of *Lactobacillus* DNA isolated from four pills are shown in Fig. 5. It follows from the results that DNA isolation from real sample was reproducible even in the presence of competitive microflora and PCR inhibitors.

4. Conclusions

The dispersion polymerization of GMA and HEMA in the presence of a ferrofluid allowed to encapsulate iron oxide in P(HEMA-GMA) microspheres, 2.9 μm in diameter with a rather narrow size distribution. The encapsulation of magnetite nanoparticles coated with oleic acid involved adsorption of the polymerization mixture components including monomer and oligomer radicals on the nanoparticles, which provided the nuclei for polymer precipitation. The coating of magnetite nanoparticles with oleic acid thus might aid the encapsulation. The microspheres were ammonolyzed and then functionalized with streptavidin using cyanuric chloride. The DNA isolated from *L. paracasei* subsp. *paracasei* CCDM and that from a real sample (BIFI-Pangamin pills) were selectively separated on magnetic P(HEMA-GMA)-NH_2-STV microspheres with immobilized biotinylated complementary DNA probe. The DNA-responsive microspheres enabled highly efficient and specific magnetic separation of target DNA from probiotic microorganisms in real samples.

Acknowledgement

This work was supported by the Ministry Education, Youth and Sports (project 2B06053), Grant Agency of the Czech Republic (projects 203/09/1242, 203/09/0857 and P503/10/0664) and EU (CaMiNEMS project 228980).

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