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Magnetic microparticulate carriers with immobilized selective ligands in DNA diagnostics

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Abstract

Magnetic poly(2-hydroxyethyl methacrylate)- and poly(glycidyl methacrylate)-based microparticles were prepared by dispersion polymerization in the presence of iron oxide nanoparticles, both commercial and laboratory-made. The polymerization was highly sensitive to even subtle changes in the various reaction parameters involved in the process. The size of the final microparticles was determined by the composition of the dispersion medium (e.g. water/ethanol ratio, monomer concentration at the moment of phase separation, stabilizer concentration, initiator type and concentration, polymerization temperature). Several DNA applications of developed microparticles were described, among others RNA and DNA degradation and *Salmonella* cell magnetic separation by RNase A and DNase I and anti-*Salmonella* or proteinase K immobilized on developed magnetic carriers. The sensitivity of polymerase chain reaction (PCR) in cell detection was negatively affected by some magnetic carriers and compounds used in their preparation. Carboxyl group-containing magnetic microparticles were prepared for isolation of genomic DNA from cell lysate in the presence of poly(ethylene glycol) and sodium chloride.

Keywords: Magnetic; DNA; Microparticles

1. Introduction

In recent years, substantial progress has been made in developing technologies in the field of magnetic carriers. They have found application in numerous biological areas, such as diagnostics, drug targeting, molecular biology, high-throughput cell and nucleic acid isolation and purification, and immunoassays [1]. Nucleic acid purification, amplification, labeling and signal detection are important especially in genome analysis. Target cells can be captured on magnetic microparticles carrying specific antibodies and separated from complex environment containing extracellular inhibitors or competitive microflora [2–4]. Target microbial cells need not be detached from magnetic microparticles and immunomagnetic separation (IMS) of cells from polymerase chain reaction (PCR) inhibitors can be effectively combined with other methods of microbial cell identification such as cultivation method (CM) or polymerase chain reaction (PCR). The inherent benefits offered by magnetic handling includes reduced reagent cost, elimination of labor-intensive steps, easy automation and high purity DNA obtained in a short time compared with conventional methods.

Many particulate carriers are based on the styrene-divinylbenzene support [5]. Its main shortage involves hydrophobic interaction with biomolecules resulting in the loss of native conformation as well as the activity of adsorbed biomolecule. Moreover, they are often produced by a very complicated and elaborated method of multistep swelling and polymerization first patented by Ugelstad [6]. Therefore, the aim of this report is to prepare magnetic microparticles with hydrophilic properties, which do not interfere with PCR, by single-step dispersion polymerization. Under appropriate conditions, this technique, which is very attractive for its simplicity, can yield magnetic particles in the micrometer size range of narrow size distribution. The advantage of hydrophilic microparticles

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consists in a low non-specific adsorption of biologically active compounds. This report is oriented on the particles from two methacrylate monomers, namely 2-hydroxyethyl and glycidyl methacrylate (HEMA and GMA). While poly(2-hydroxyethyl methacrylate) (PHEMA) is well known for its non-toxicity, biocompatibility and widespread use in biomedical applications, oxirane groups of poly(glycidyl methacrylate) (PGMA) can be readily modified (hydrolysis, ammonolysis, oxidation, etc.). Several applications of the microparticles are presented.

2. Experimental

2.1. Chemicals

HEMA, GMA (both Röhm GmbH, Germany) and ethylene dimethacrylate (EDMA; Ugilor, France) was vacuum distilled before use. 2,2′-Azobisisobutyronitrile (AIBN, Fluka) was twice recrystallized from ethanol. Dibenzoyl peroxide (BPO), $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$ were purchased from Fluka and used without further purification. The following stabilizers were used: poly(ethylene glycol) (PEG; $M_w = 6000$; Serva), poly(vinylpyrrolidone) PVP K 15 and K 30 ($M_w = 10{,}000$ and 40,000; Fluka), cellulose acetate butyrate (CAB; acetyl/butyryl 35/15, $M_n = 100{,}000$; Eastman, USA). All other chemicals were supplied from Aldrich and used as received.

2.2. Preparation of microparticles

Typical recipes used for preparation of P(HEMA-*co*-EDMA) and PGMA microparticles are shown in Table 1. Steric stabilizer (cellulose acetate butyrate or poly(vinylpyrrolidone)) was first dissolved in a solvent containing iron oxide, a solution of an initiator in monomer(s) was added and the mixture bubbled with nitrogen for 10 min. The polymerization was run in a 100 ml reactor equipped with an anchor-type stirrer (500 rpm) for 17 h at 70 °C. After completion of the polymerization, some blank (non-magnetic) microspheres were easily removed by supernatant replacement during purification by repeated magnetic separation from toluene (P(HEMA-*co*-EDMA) microparticles) or ethanol (PGMA microparticles). While magnetic particles concentrated at the bottom, the upper layer was easily decanted. The procedure was repeated (up to 20 times) until excess stabilizer was removed and no more solid was in the supernatant.

Table 1
Standard recipes for preparation of magnetic P(HEMA-*co*-EDMA) and PGMA microparticles by dispersion polymerization

Component	Weight (g)	Component	Weight (g)
HEMA	11.04	GMA	12
EDMA	0.96	PVP	2.4
CAB	3.2	Ferrofluid[a]	1
Ferrofluid[a]	1	AIBN	0.24
BPO	0.24	Ethanol	68
Toluene/2-methylpropan-1-ol	22.2/45.8		

At 70 °C, 16 h.
[a] Dry weight.

2.3. Oxidation of P(HEMA-co-EDMA) microparticles

Hydroxy groups of magnetic P(HEMA-*co*-EDMA) particles containing needle-like maghemite (1 g) were oxidized to carboxyl ones with 2 wt% aqueous solution of potassium permanganate (20 ml) for 3 h at room temperature under acid conditions (1 M sulfuric acid; 20 ml) [7] in the presence of a small amount (10 mg) of an alkylbenzenesulfonic acid wetting agent (Abeson K; Spolchemie Ústín/Labem, Czech Republic). After careful washing with 10 wt% of potassium disulfite, hydrochloric acid, water and freeze-drying, the content of COOH groups determined by potentiometric titration after ion exchange with barium chloride [8] was 0.85 mmol/g (hydrolyzed PGMA containing PEG-stabilized magnetite even 2.6 mmol COOH/g).

2.4. Preparation of lanthanide complexes

A chelating macroporous copolymer with ethylenediaminetetraacetic acid (EDTA) ligands was prepared from P(GMA-*co*-EDMA) (60/40) particles [9], 4–8 μm in size, by a two-step reaction. First step consisted of ammonolysis of oxirane groups with aqueous ammonia for 2 days at room temperature [10]. The amino sorbent contained 1.67 mmol $-NH_2$/g of dry substance (according to nitrogen analysis). Amino derivative (1 g) was suspended in 10 ml of anhydrous pyridine, refluxed under stirring with EDTA dianhydride (2.05 g) [11] for 2 h and then left standing overnight at room temperature. The product was washed with water and the remaining anhydride groups were hydrolyzed under mild conditions. According to nitrogen analysis the carrier contained 0.48 mmol EDTA/g of dry substance.

The cleavage activity of the immobilized system was assayed after incubation of 10 μl of a water suspension containing 0.1 mg carrier, 0.2 μl plasmid DNA (1 μg/μl) and 4 μl 50 mM HEPES buffer. The volume of the reaction mixture was adjusted to 20 μl with water. Plasmid pBR322 DNA was treated at 76 °C. The reaction time was up to 10 h. The reaction was stopped by cooling and gel electrophoresis analysis was carried out immediately.

2.5. Immobilization technique

To attach *Salmonella* antibodies and proteinase K, magnetic P(HEMA-*co*-EDMA) and hydrolyzed (0.05 M sulfuric acid, 5 h/50 °C) P(HEMA-*co*-GMA) microspheres were activated with 2,4,6-trichloro-1,3,5-triazine in acetone and the respective proteins added in 0.05 M phosphate

buffer (pH 8) with 0.15 M NaCl. The experimental conditions for immobilization were based on a previously published procedure [12]. The immobilization itself proceeded at pH 8 and 23 °C for 4 h. The product was repeatedly washed with water and lyophilized.

Magnetic P(HEMA-*co*-EDMA) microspheres contained 3.1 mg antibody/g or 10.2 mg proteinase K/g of carrier, and magnetic P(HEMA-*co*-GMA) particles contained 3.0 mg antibody/g of carrier. The amount of antibody and/or enzyme bound to the matrix was determined from the difference of concentrations in the reaction solution before and after the coupling using UV absorption at 280 nm.

2.6. Cell cultivation and DNA isolation

Bacterial cells of *Bifidobacterium longum* were anaerobically cultivated on a MRS medium (Oxoid) containing cysteine (0.5 g/l) overnight (18 h). Total of 1 ml of cells was washed and resuspended in 100 µl lysis buffer (10 mM Tris–HCl, pH 7.8; 5 mM EDTA, pH 8.0; lysozyme 0.3 mg/ml); 10 µl proteinase K (10 mg/ml) and 2.5 µl SDS (20%) were then added and the mixture incubated for 18 h at 55 °C. DNA was extracted from the crude cell lysates by phenol [13] (control experiment). Typically, a total of 50 µl of crude cell lysate, 10 µl of magnetic P(HEMA-*co*-EDMA)-COOH particle suspension and 50 µl of hybridization buffer (20% PEG, 2.5 M NaCl) was mixed and incubated for 10 min at laboratory temperature. The final concentrations of PEG and NaCl were 9.1% and 1.1 M, respectively, which were suitable conditions for DNA adsorption. If crude cell lysates were used, the phenol extraction step was omitted. DNA was adsorbed directly from cell lysates on microparticles and the eluted DNA was used in PCR. The microparticles with adsorbed DNA were then magnetically separated, washed twice with 200 µl of 70% ethanol and shortly dried. Particles with DNA were eluted in 50 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.8). DNA in the eluate (2 µl) was used as DNA matrix in PCR amplification. The nucleic acid identity was checked by gel electrophoresis and purity confirmed by UV spectrophotometry at 260 and 280 nm [14].

2.7. PCR amplification and detection of PCR products

DNA purified by phenol extraction [13] or DNA eluted from microparticles was used as a matrix in PCR and PbiF1 and PbiR2 as primers which make it possible to amplify 914 bp long DNA fragment specific to the *Bifidobacterium* genus [15]. Typically, the PCR mixture contained 1 µl of each 10 mM dNTP, 0.5 µl (10 pmol/µl) each primer, 1 µl DNA matrix, and 1 µl RecTaq polymerase 1.1 (1 U/µl), 2.5 µl buffer; PCR water was added to 25 µl volume. After 5 min of the initial denaturation period at 94 °C (hot start), amplification was carried out in 30 cycles of 60 s at 94 °C, 60 s at 50 °C, and 120 s at 72 °C. In the last cycle, the elongation step at 72 °C was prolonged to 10 min. The PCR products were detected using agarose gel electrophoresis in 1.2% agarose gel in TBE buffer (45 mM boric acid, 45 mM Tris–base, 1 mM EDTA, pH 8.0). DNA was stained with ethidium bromide (0.5 µg/ml), decolorized in water, and photographed in 305 nm UV light on a TT667 film. The lengths of amplified DNA fragments were calculated using the Anagel program [16].

3. Results and discussion

3.1. Colloidal iron oxide

Superparamagnetic colloidal magnetite to be incorporated into the microparticles during subsequent polymerization was obtained by chemical coprecipitation of Fe^{2+} and Fe^{3+} salts with ammonia [17]. Several stabilization modes to prevent coalescence, namely coating with compounds such as oleic acid, poly(ethylene glycol) (PEG) and poly(vinylpyrrolidone) (PVP), which stabilized magnetite sterically, and also electrostatic stabilization taking advantage of ionic charges on the particle surface, were compared. The above compounds influenced also properties of resulting magnetic microparticles, e.g. colloidal stability, morphology, size and polydispersity (Table 2). Electrostatic stabilization of magnetite in water/ethanol was achieved by its treatment with either perchloric acid or tetramethylammonium hydroxide (TMAH) [13]. Oleic acid-coated magnetite nanoparticles, mostly in the 10 ± 5 nm diameter range, are shown in Fig. 1(a), their electrostatically stabilized counterparts, which did not substantially differ in size from that of the oleic acid-coated ones, are in Fig. 1(b). Good dispersibility of oleic acid-coated nanoparticles in non-polar solvents is a result of their hydrophobization. Coating of colloidal magnetite with oleic acid appeared to be suitable for toluene/2-methylpropan-1-ol medium. It is just to remind that while the electrostatically stabilized magnetite formed ferrofluid in ethanol, oleic acid-coated magnetite coagulated there.

3.2. Magnetic microspheres

Superparamagnetic colloids (cores) were encapsulated in polymer microparticles by dispersion polymerization of HEMA or GMA or copolymerization of both monomers. Mechanism of dispersion polymerization consisting of nucleation and growth stage was described elsewhere [18]. A prerequisite for dispersion polymerization is the use of the solvent which dissolves the monomer(s), stabilizer, initiator, but not the polymer formed. While dispersion polymerization of HEMA or its copolymerization with GMA had to proceed in a rather low-polar toluene/2-methylpropan-1-ol medium [19,20] characterized by the solubility parameter values of the polymerization mixture ranging from ca. 18–21 $MPa^{1/2}$, dispersion polymerization of more hydrophobic GMA necessitated ethanol (or its

Table 2
Characterization of prepared magnetic polymer microparticles

Component	Encapsulated iron oxide	Fe content (wt%)	Oxirane content (mmol/g)	Diameter (μm)	Polydispersity index[a]
P(HEMA-*co*-EDMA) (92/8)	Needle-like maghemite	7.5	–	2.1	1.11
P(HEMA-*co*-GMA) (1/1)	Oleic acid-coated magnetite	5.3	3.8	1.7	1.05
PGMA	$HClO_4$-treated magnetite	12.9	1.2	0.36	1.04
PGMA	TMAH-treated magnetite	5.9	1.4	0.74	1.07

[a] Polydispersity index—weight- to number-average particle diameter.

aqueous solution) with the solubility parameter of the reaction mixture around 25 $MPa^{1/2}$ [21]. In the dispersion polymerization, stabilization of mature microparticles plays a crucial role in prevention of their aggregation determining the final microparticle size. This was achieved by using cellulose acetate butyrate (M_n = 100,000; 35/15 acetyl/butyryl) in toluene/2-methylpropan-1-ol and by poly(vinylpyrrolidone) K 30 (M_w = 40,000) in ethanolic medium. Selection of a proper polymerization initiator is no less important. While dibenzoyl peroxide (BPO) was optimal for (co)polymerization of HEMA, 2,2′-azobisisobutyronitrile (AIBN) was the best for dispersion polymerization of GMA in ethanol. Also ammonium persulfate (APS) was tested as initiator producing truly fine

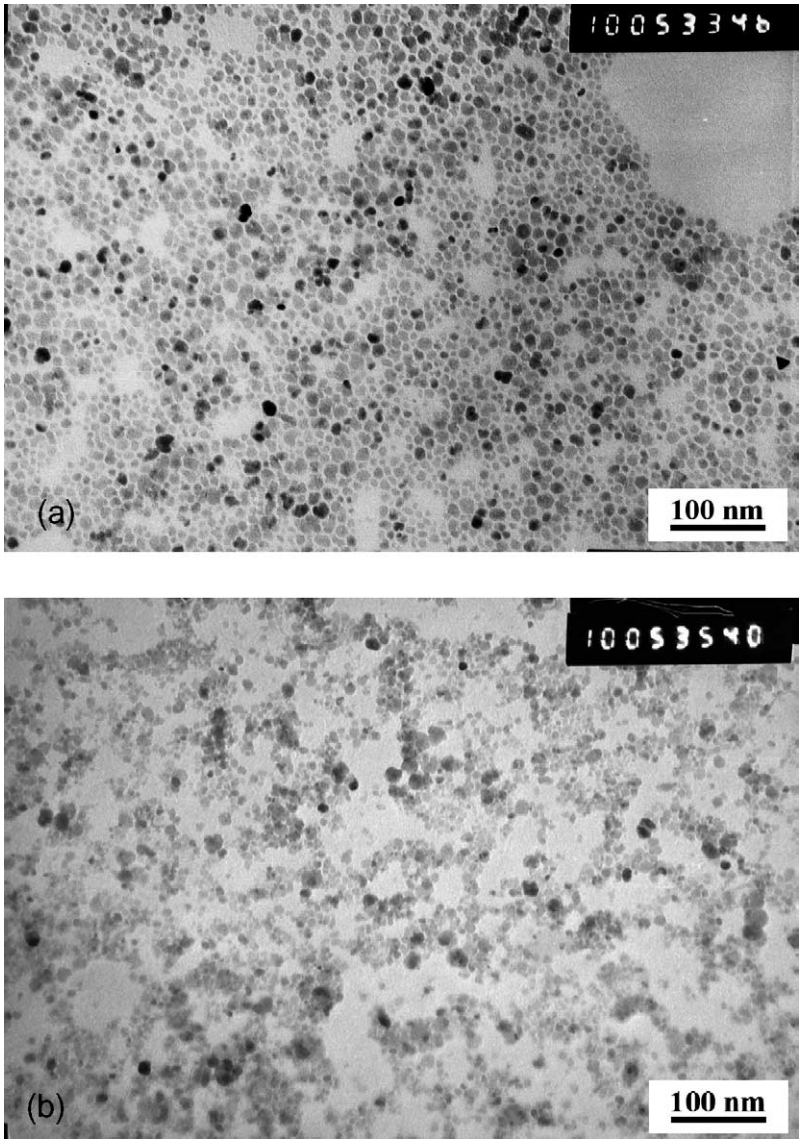

Fig. 1. TEM of colloidal magnetite stabilized with (a) oleic acid (spread from toluene), (b) $HClO_4$ (spread from water).

nanoparticles; their broad particle size distribution was, however, a disadvantage. In this report, the basic recipes given in Table 1 were used. Typically, one parameter was varied as specified while keeping the others the same as given in the recipe. Both the concentration of monomer and stabilizer was thus kept constant at 15 and 3 wt%, respectively, based on total weight. In contrast, the AIBN initiator concentration was related to the monomer and amounted to 2 wt%; the weight ratio of magnetite nanoparticles to the monomer was mostly 0.08 (or 0.05). The resulting hybrid microparticles were characterized by determining their size and size distribution, the content of iron and oxirane groups (Table 2). Magnetic P(HEMA-*co*-EDMA) and P(HEMA-*co*-GMA) microparticles 2.1 and 1.7 μm in diameter obtained in the presence of needle-like maghemite and oleic acid-coated magnetite (ferrofluid) are shown in Fig. 2(a) and (b), respectively. If colloidal magnetite was treated with PEG 6,000 or PVP K 15, it had a tendency to agglomerate and was apparently not completely encapsulated in polymer microparticles [20]. Interestingly, addition of colloidal iron oxide in the polymerization feed induced always a substantial decrease in PGMA microparticle size due to electrostatic charges on colloidal iron oxides, improving thus particle stabilization (Fig. 2(c) and (d)). However, nonencapsulated iron oxide nanoparticles were present in the resulting polymer microparticles if the weight ratio of Fe_3O_4 to GMA in the feed exceeded 0.05. Excessive iron oxide can be removed by washing with 2 M HCl, but this is associated with hydrolysis of oxirane groups. Moreover, addition of colloidal iron oxide to the polymerization feed induced particle size distribution broadening. This broadening indicates prolongation of nucleation stage, which is probably a result of inhibition of polymerization due to the reaction of iron ions with radicals from the initiator. It is just to remind that a sharp particle size distribution is an indication that the initial nuclei were formed within a very short time period and subsequent growth took place without formation of new nuclei and without agglomeration of particles. It may be thus concluded that it is very difficult to obtain large microparticles with a high iron oxide content in a high yield.

The prepared magnetic microparticles can be used for immobilization of biomolecules (enzymes, proteins, antibodies). Steric accessibility of immobilized ligands (proteins) could be, however, reduced by aggregation of magnetic PHEMA microparticles (even if crosslinked with EDMA) occurring in water media, which would disqualify their medicinal-biological application. This shortcoming

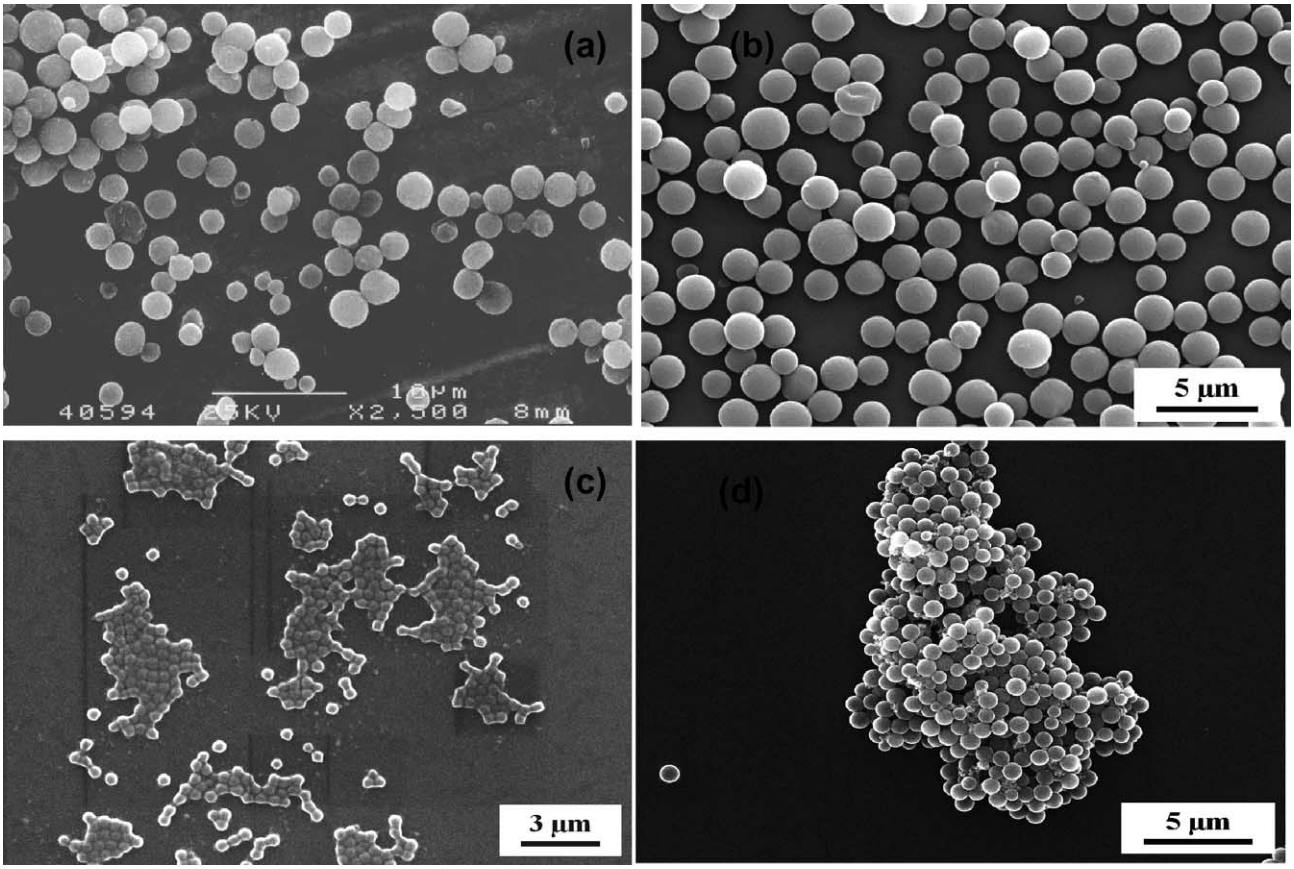

Fig. 2. SEM of magnetic (a) 92/8 P(HEMA-*co*-EDMA) containing needle-like maghemite, (b) 1/1 P(HEMA-*co*-GMA) containing oleic acid-coated Fe_3O_4 and PGMA containing electrostatically- (c) $HClO_4$- and (d) TMAH-stabilized Fe_3O_4.

was obviated in magnetic P(HEMA-*co*-GMA) microparticles and even better in magnetic PGMA microparticles which do not have any tendency to agglomerate in water. Moreover, their oxirane groups easily undergo hydrolysis, oxidation to aldehyde, transformation to amine or any other group necessary for immobilization of a target biologically active compound. Magnetic PGMA microparticles encapsulating electrostatically stabilized colloidal magnetite (Table 2) are shown in Fig. 2(c) and (d). The only problem is whether the oxirane groups are able to completely survive the polymerization. This answer is positive for PGMA microparticles obtained with colloids precipitated in the absence of electrostatic stabilizing layers during iron oxide preparation. The content of oxirane groups in the resulting microparticles, as determined by IR spectroscopy from a characteristic band at 906–908 cm^{-1}, reached at the minimum 6 mmol/g, i.e. virtually coincided with the theoretically expected amount under the assumption of no side reactions occurring during the polymerization. However, content of oxirane groups was rather low when magnetite nanoparticles in the microparticles were treated with perchloric acid or tetramethylammonium hydroxide (Fig. 3). It reached 1.2 and 1.4 mmol of oxirane groups per gram of polymer if cationic and anionic ferrofluid was used, respectively (Table 2). $HClO_4$ or TMAH obviously catalyzed hydrolysis of oxirane groups or their reaction with ethanol.

There are several possibilities of controlling the PGMA particle size and its distribution by variation of reaction parameters. Probably the most important one takes advantage of the dependence of particle size on polarity of the reaction medium. The polarity modifies the operation (solubility) of stabilizer because polarity of the medium changes the interaction of stabilizer and the formed particles. The polarity also affects nucleation. The solvency determines the critical molecular weight, above which the polymer precipitates, and ultimately, the final particle size. The more soluble the polymer, the later it precipitates in a smaller number of nuclei and, correspondingly, larger particles are formed. In the dispersion polymerization of GMA in the presence of colloidal iron oxide, mixtures of low- and high-solubility-parameter solvent, ethanol ($\delta = 26\ MPa^{1/2}$) and water ($\delta = 47.9\ MPa^{1/2}$), respectively, allowed the reaction solvency to be adjusted by varying the ratio of both solvents. The polarity of the mixture (reflecting the degree of hydrophilicity) increased with increasing solubility parameter. The dependence of PGMA microsphere diameter on the solubility parameter passed through a maximum at about 10 wt% of water ($27.9\ MPa^{1/2}$)—see Fig. 4. This maximum, which is in agreement with previously published results on dispersion polymerization of GMA in the absence of ferrofluid [21], can be ascribed to an anomalous thermodynamics as a result of incomplete mixing of alcohol and water at the molecular level [22]. Fig. 4 also documents the reduced particle size of magnetic versus nonmagnetic PGMA microparticles. Another important parameter to control particle size is concentration of the stabilizer. As the concentration of PVP stabilizer increased, there was an increase in the number of stable nuclei owing to a larger surface area for stabilization, so correspondingly smaller PGMA particles were produced (Fig. 5). Other parameters influencing the particle size are monomer concentration and polymerization temperature. With increasing value of both variables, PGMA microparticle size increased due to higher polymer solubility [20].

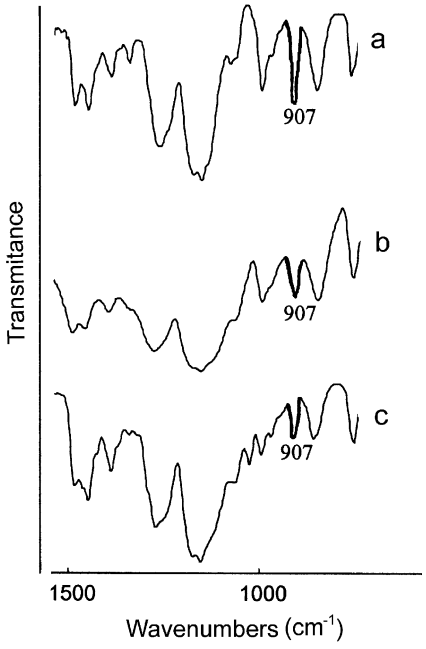

Fig. 3. IR spectrum of magnetic PGMA microparticles prepared in the presence of (a) bare, (b) $HClO_4$- and (c) TMAH-treated ferrofluid.

3.3. Immobilized RNase A

Chromatography is an integral part of purification of plasmid vectors. To achieve a good resolution of plasmid

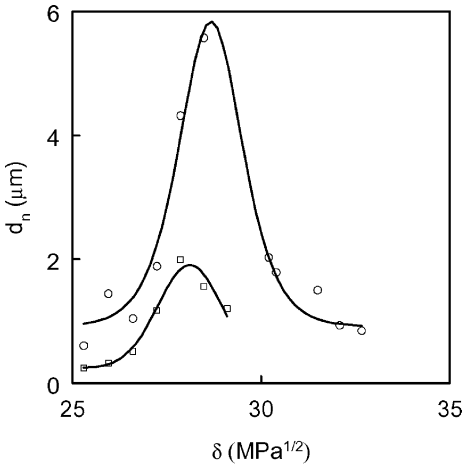

Fig. 4. Dependence of (□) magnetic and (○) non-magnetic PGMA microsphere diameter d_n on solubility parameter δ.

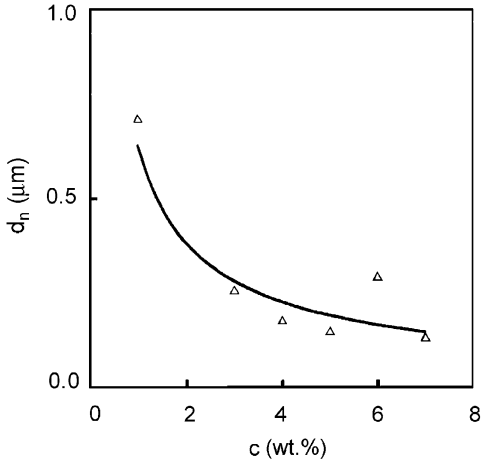

Fig. 5. Dependence of magnetic PGMA microparticle diameter d_n on PVP stabilizer concentration c.

DNA in size-exclusion chromatography, it is necessary to degrade high-molecular-weight RNA by RNase A. If RNase A is immobilized to a suitable magnetic carrier, it can be easily removed from the reaction mixture with a simple magnetic separator and, moreover, it can be used repeatedly. RNase A was therefore coupled to magnetic P(HEMA-*co*-EDMA) microparticles by the 2,4,6-trichloro-1,3,5-triazine method [12] and its relative activity estimated in temperature range 30–80 °C and pH 4.0–8.0. Maximum activity was observed at 70 °C and pH 6–6.5 [23]. Immobilized RNase A was thermally stable at 30–60 °C. Gel electrophoresis of plasmid DNA pUC 19 contaminated with bacterial RNA confirmed RNA degradation with the immobilized enzyme (Fig. 6).

3.4. Immobilized DNase I

DNase I is often used to introduce single- or double-stranded nicks into DNA chains [13] and for gene expression by the reverse transcription polymerase chain reaction (RT-PCR) [24]. Depending on the metal ion used as activator, DNase I hydrolyses single- or double-stranded DNA [25,26]. DNase I immobilized on magnetic P(HEMA-*co*-EDMA) microparticles degraded chromosomal and plasmid DNAs in the presence of divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+}) used for the activation [27]. Activity of free and magnetic particle-immobilized enzyme, depending on pH and activating cation, was examined. Compared with free DNase I, the maximum activity of immobilized DNase I was shifted to lower pH. DNase I immobilized on magnetic particles was used repeatedly in the degradation of chromosomal DNA. Its residual activity was influenced by the nature of activating divalent cation [27]. When activity of immobilized enzyme (activated by Mg^{2+} or Ca^{2+} ions) decreased, it was reactivated with Co^{2+} ions.

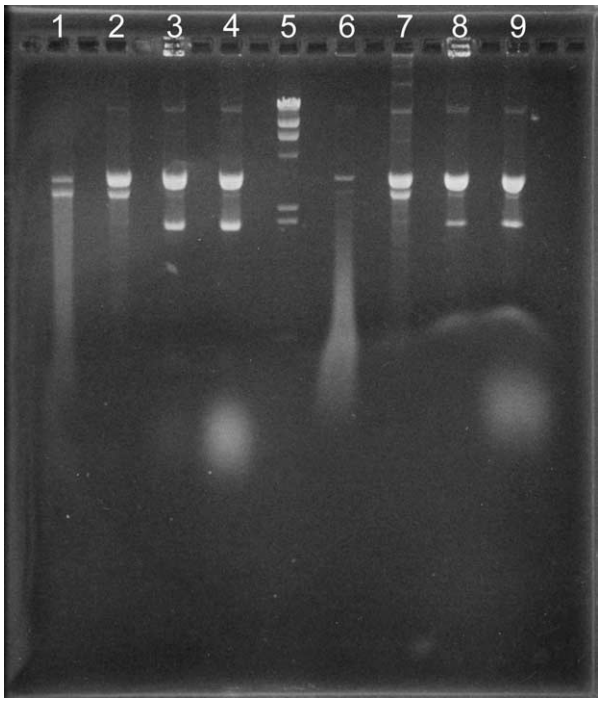

Fig. 6. Agarose gel electrophoresis of plasmid DNA pUC 19 (contaminated by bacterial RNA) confirmed RNA degradation with RNase A immobilized on magnetic P(HEMA-*co*-EDMA) microparticles. Lanes 1–4 and 6–8: crude lysates digested for 1 and 3 h, respectively; lanes 1 and 6: free RNase A; lanes 2 and 7: free RNase A with DNase activity reduced by boiling; lanes 3 and 8: RNase A immobilized on magnetic P(HEMA-*co*-EDMA) microparticles; lanes 4 and 9: controls without RNase A; lane 5: λ DNA/Hind III standard.

3.5. Immobilized anti-Salmonella

PCR determination of *Salmonella* relies on analysis of DNA. Recently, commercially available anti-*Salmonella* magnetic particles were found suitable for separation of *Salmonella* cells [5]. Immunomagnetic separation—PCR (IMS-PCR) was successfully used for identification and fast confirmation of nontypical *Salmonella* strains isolated from human stool and rabbit meat [28]. As the recently used particles are often hydrophobic, magnetic hydrophilic nonporous P(HEMA-*co*-EDMA) microparticles functionalized with polyclonal *Salmonella* antibodies were proposed for IMS of *Salmonella* cells [29]. The cells were then identified by cultivation method (CM) and PCR. IMS of cells thus solved problems associated with falsely negative PCR results caused by PCR inhibitors present in processed food products, e.g. milk powder and dried eggs. Operational suitability of newly designed P(HEMA-*co*-EDMA) microparticles with immobilized *Salmonella* antibodies was confirmed by IMS of *Salmonella* cells for 15 min at 24 °C, which was followed by cultivation and PCR identification [29]. IMS, however, cannot eliminate intracellular PCR inhibitors present in immunoseparated *Salmonella* cells isolated from field samples.

3.6. Immobilized proteinase K

Magnetic P(HEMA-*co*-EDMA) microparticles with immobilized proteinase K were investigated for degradation of intracellular protein inhibitors present in *Salmonella* cells. A negative influence of PCR inhibitors in the *Salmonella* arizonae 18/78 strain on PCR was eliminated by treating the cells with proteinase K immobilized on magnetic P(HEMA-*co*-EDMA) microparticles at 55 °C for 1 h [29]. Particles with the immobilized enzyme could be easily removed from the reaction mixture with a magnet and the laborious removal of free enzyme and/or enzyme inhibitor was thus avoided.

3.7. Effect of components on PCR

PCR assays became recently indispensable for fast and sensitive detection of various DNAs in raw samples, however, intracellular and intracellular inhibitors are often interfering. Magnetic particles are therefore suitable for inhibitor-free separation of cells or DNA [30]. The particles, however, must not interfere with PCR, the sensitivity of which for target DNA detection could be negatively influenced by the presence of some compounds used in their preparation. The influence of both non-magnetic and magnetic P(HEMA-*co*-EDMA) and PGMA microparticles, including magnetite colloids and various components used in their preparation, on PCR was investigated using DNA isolated from G^+ bacteria *Bifidobacterium longum* [31]. Different concentrations of DNA mixed with tested compounds were examined with and without magnetic separation. The presence of interfering components manifested itself by a decrease in PCR sensitivity (only higher DNA concentrations gave a detectable PCR product), or by falsely negative results. PCR was not affected in the presence of non-magnetic, in contrast to some magnetic carriers. PCR sensitivity decreased after addition of magnetic PHEMA core-shell microcarriers containing needle-like maghemite or oleic acid-coated magnetite core in PCR mixture (Table 3). As PCR sensitivity was evidently negatively influenced by the presence of some compounds used in preparation of magnetic particles, their effect on PCR was investigated. The results are given in Table 4, from which it is clear that the most interfering compounds are needle-like maghemite or oleic acid-, PVP- and PEG-treated colloidal magnetite. PEG or PVP did not probably capture on the magnetite surface and bare iron ions in its crystal lattice inhibited the reaction. Fig. 7 then shows agarose gel electrophoresis of PCR products after amplification of *Bifidobacterium bifidum* DNA in the presence of oleic acid-stabilized magnetite documenting thus its interference. Oleic acid-stabilized magnetite in high concentrations interfered with PCR (no PCR products were detected—see lanes 7–10 in Fig. 7) without magnetic separation. However, the same concentrations of nanoparticles magnetically separated did not interfere in PCR. Low magnetite concentrations did not interfere in PCR, either with or without the separation (see lanes 11–14 in Fig. 7).

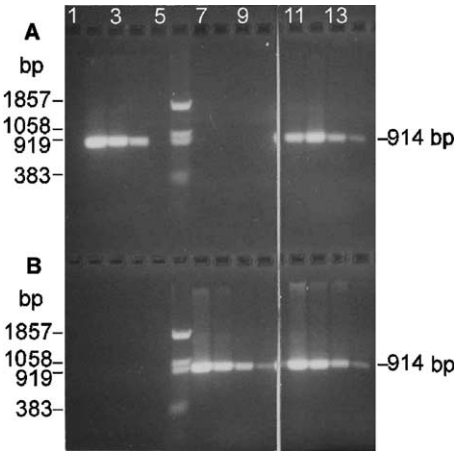

Fig. 7. Agarose gel electorphoresis of PCR products obtained after amplification of *Bifidobacterium bifidum* DNA in the presence of oleic acid-coated magnetite. Without magnetic separation (A)—lane 1: negative control without DNA; lanes 2–5: control with 2, 200, 20 and 2 pg of DNA; lane 6: DNA standard (pBR 322/BstNI); lanes 7–10: PCR mixtures containing magnetite (5 μg/25 μl PCR mixture) (undiluted); lanes 11–14: PCR mixtures containing magnetite (100×diluted (50 ng/25 μl PCR mixture)). With magnetic separation (B)—lanes 1–5: without DNA; lanes 6–14: the same as in A.

The reason why all magnetite particles partially inhibited PCR or gave falsely negative results could consist in the presence of ferric ions in the solution, which was experimentally confirmed at different concentration of Fe^{3+} ions in PCR mixture ($0.1–1\times10^{-12}$ mM)—see Fig. 8. Concentration 0.1 μM Fe^{3+} ions and higher inhibited PCR. Equilibrium between Fe^{3+} ions in magnetite nanoparticles and in the solution is namely dynamic, i.e. Fe^{3+} ions can cross from the solid phase to solution and vice versa. The assumption was further proved by a moderate inhibition of PCR in the presence of supernatant

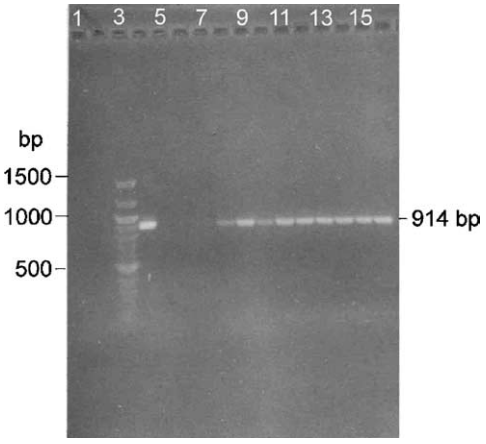

Fig. 8. Agarose gel electrophoresis of PCR products obtained after amplification of *Bifidobacterium bifidum* DNA in the presence of $FeCl_3$ ($0.1–1\times10^{-12}$ mM). Lanes 1–2: controls without DNA; lane 3: DNA standards—100 bp ladder; lane 4: positive control with 2 ng of DNA; lanes 5–16: PCR mixture containing $0.1–1\times10^{-12}$ mM $FeCl_3$.

Table 3
Effect of non-magnetic P(HEMA-*co*-EDMA) and magnetic P(HEMA-*co*-EDMA) and PGMA microparticles on PCR

Component	Component/PCR mixture (μg/μl)	*Bifidobacterium bifidum* DNA/PCR (pg/25 μl) PCR product[a]			
		2000	200	20	2
–	0	++	++	+	+/−, −
Nonmagnetic P(HEMA-*co*-EDMA)	200	++	+	+	+/−
Magnetic P(HEMA-*co*-EDMA)[b]	200	−	−	−	−
	20	++	+	+/−	−
	2	++	+	+	−
	0.2	+++	++	+	+/−
Magnetic PGMA[c]	200	+++	++	+	+/−
Magnetic PGMA[d]	200	+++	++	+	+/−

[a] PCR: +++, ++, +, +/− denote bands of very high, high, good and weak intensity, respectively; − no band.
[b] Needle-like γ-Fe_2O_3 encapsulated in the microparticles.
[c] $HClO_4$-treated Fe_3O_4 encapsulated in the microparticles.
[d] TMAH-treated Fe_3O_4 encapsulated in the microparticles.

remaining after removal of PEG- or PVP-coated magnetite nanoparticles on a magnetic separator. Another explanation may consist in the interaction with the exposed surface iron ions in the lattice. Nevertheless, it can be concluded that in addition to magnetite, PCR was inhibited also by other components used in the nanoparticle synthesis, especially by PEG (Table 4).

Based on the above results, magnetic PGMA particles encapsulating electrostatically stabilized colloidal magnetite were investigated (Table 2). Surprisingly, they did not interfere with the PCR course (Table 3), no matter what charge was introduced to prevent colloid aggregation: whether positive (using perchloric acid) or negative (using tetramethylammonium hydroxide). Agarose gel electrophoresis of PCR products obtained after amplification of *B. bifidum* DNA in the presence of magnetic PGMA microparticles encapsulating perchloric acid-coated magnetite is shown in Fig. 9. It also confirmed that the microparticles added to the PCR mixture did not interfere with PCR, in contrast to, e.g. P(HEMA-*co*-EDMA) microparticles prepared in the presence of needle-like maghemite. Magnetic PGMA microparticles prepared in the presence of electrostatically-stabilized iron oxide did not aggregate in water solutions nor detectably interfered with PCR (Table 3,

Table 4
Effect of components used in magnetite preparation on PCR

Component	Component/PCR mixture (μg/25 μl)	Separation	*Bifidobacterium bifidum* DNA/PCR mixture (pg/25 μl) PCR product[a]			
			2000	200	20	2
–	0	[b]	+++	++	++	+/−, −
Needle-like γ-Fe_2O_3	5	[b]	+++	+	+/−	−
	5×10^{-2}	[b]	+++	+++	++	−
	5	[c]	+++	+++	+	+/−
Oleic acid	5	[b]	+++	+/−	+/−	−
	5×10^{-2}	[b]	+++	++	+	+/−
PVP K 15	5	[b]	+++	++	+	+/−
PEG 6,000	5	[b]	+++	−**	−**	−**
	5×10^{-2}	[b]	+++	++	+	−
TMAH[d]	5	[b]	+++	++	++	+
$HClO_4$	5×10^{-3}	[b]	−**	−**	−**	−**
Fe_3O_4 (oleic acid[e])	5	[b]	+++	−**	−**	−**
Fe_3O_4 (PVP K 15[e])	5	[b]	−**	−**	−**	−**
	5×10^{-2}	[b]	++	+	+/−	−
	5	[c]	+	−**	−**	−**
	5×10^{-2}	[c]	+++	++	+	−
Fe_3O_4 (PEG 6,000[e])	5	[b]	−**	−**	−**	−**
	5×10^{-2}	[b]	++	+	+/−	−
	5	[c]	−**	−**	−**	−**
	5×10^{-2}	[c]	+++	++	+	+/−

[a] PCR: +++, ++, +, +/− denote band of very strong, strong, mean and weak intensity, respectively; − no band; **falsely negative result.
[b] Without magnetic separation.
[c] With magnetic separation.
[d] TMAH, tetramethylammonium hydroxide.
[e] Coating.

Fig. 9) even if perchloric acid itself interfered with the PCR course (Table 4). This indicates that colloidal magnetite was completely incorporated inside the PGMA microspheres. In contrast, it can be speculated that PEG 6,000 or PVP K 15 did not completely coat magnetite which was then just on the surface of polymer microparticles negatively thus affecting the sensitivity of PCR approximately in the same way as magnetic P(HEMA-*co*-EDMA) microparticles containing needle-like maghemite (Tables 3 and 4). Study of the effect of components on PCR enabled thus verification of the incorporation of magnetite nanoparticles in the particular methacrylate-based polymer microparticles and evaluation of suitability of their application in PCR. Electrostatically stabilized colloidal magnetite can be recommended in the design of new magnetic methacrylate-based microparticles obtained by dispersion polymerization because it ensures its complete encapsulation.

3.8. Carboxyl group-containing microparticles

The presence of DNA polymerase inhibitors in samples such as polysaccharides, phenolic compounds or humic acids can affect the reliability of PCR. Both magnetic and non-magnetic DNA-specific microparticles successfully eliminated the presence of inhibitors which prevent PCR amplification of extracted DNA according to different protocols. Recently, a high-throughput genome isolation has been developed in automated format based on the solid phase reversible immobilization (SPRI) in the presence of NaCl and PEG. Using this protocol, high-grade DNA template was isolated on carboxyl group-containing magnetic particles [32]. In this report, the solid-phase technique took advantage of magnetic weakly acid cation exchange derivatives of P(HEMA-*co*-EDMA) and P(HEMA-*co*-GMA) microparticles for isolation of genomic DNA from cell lysate. Up to ca. 2.6 mmol of carboxyl groups per gram of carrier was introduced into the microparticles by oxidation of hydroxy groups with a strong oxidation agent—potassium permanganate. The resulting particles reversibly bound DNA in the presence of PEG and sodium chloride. Various PEG and sodium chloride concentrations were therefore investigated to optimize adsorption of genomic DNA isolated from *Bifidobacterium* cells. Significant adsorption was achieved at PEG and NaCl concentrations higher than 5 and 1 wt% (relative to the sample volume), respectively. The more carboxyl groups on the microparticles, the higher was DNA adsorption. DNA quality was confirmed in PCR. DNA released from the microparticles served as a PCR matrix and it was amplified under the formation of a specific PCR product (Fig. 10). The eluted amount of DNA was sufficient for PCR identification of *Bifidobacterium* cells. Tested particles were used for isolation of DNA from different dairy products (butter milk, cheese, yoghurt) containing *Bifidobacterium* cells.

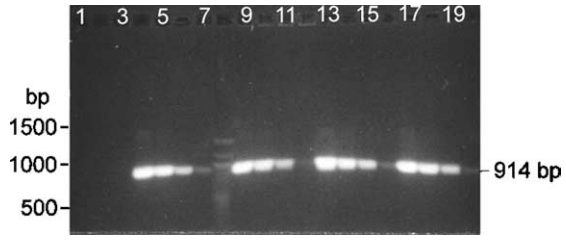

Fig. 9. Agarose gel electrophoresis of PCR products obtained after amplification of *Bifidobacterium bifidum* DNA in the presence of magnetic PGMA microparticles containing $HClO_4$-stabilized Fe_3O_4 (without magnetic separation). Lanes 1–3: negative controls without DNA; lanes 4–7: controls with 2, 200, 20 and 2 pg of DNA dissolved in water; lane 8: DNA standards—100 bp ladder; lanes 9–12, 13–16 and 17–20: DNA (2, 200, 20 and 2 pg) amplified in the presence of tested particles (undiluted, diluted 10× and 100×).

3.9. Immobilized lanthanide complexes

Development of synthetic, sequence-selective cleavage agents and structure probes for DNA and DNA-bound drugs is essential for further anticipated applications in molecular biology, medicine and related fields. In particular, lanthanides are excellent catalysts of DNA cleavage [33]. They hydrolyze phosphodiester linkages in simple phosphate esters, oligonucleotides, RNA and single-stranded DNA under physiological conditions at reasonable rates. Heterogeneous lanthanide complexes (Eu^{3+}, Gd^{3+}, La^{3+}, Nd^{3+} and Pr^{3+}) of ethylenediaminetetraacetic acid (EDTA) immobilized on insoluble PGMA support were used as catalysts of pBR322 DNA cleavage (Fig. 11). Formation of linear (double-stranded break) and nicked plasmid (single-stranded break) was investigated in dependence on the reaction conditions. Both forms were also found to depend on reaction time [34]. Also temperature of reaction mixture had a substantial influence on the cleavage rate. While the oc form increased as a result of single-stranded break, the ccc form increased due to double-stranded break.

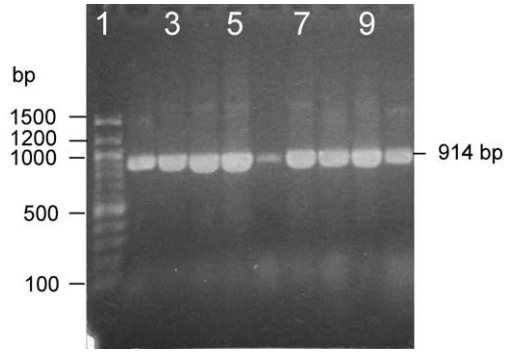

Fig. 10. Agarose gel electrophoresis of PCR products obtained after amplification of different strains of *Bifidobacterium bifidum* DNA eluted from P(HEMA-*co*-EDMA)-COOH microparticles containing needle-like maghemite. Lane 1: DNA standard (100 bp ladder); lanes 2–10: specific PCR products from different strains of *B. bifidum*.

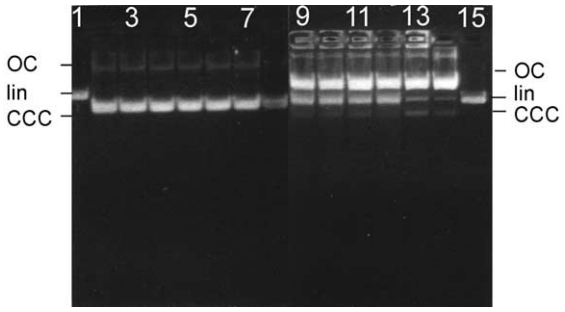

Fig. 11. Agarose gel electrophoresis of pBR322 DNA cleaved by lanthanide EDTA-immobilized on PGMA particles. Lanes 2–8: control—pBR322 DNA without carrier; lanes 9–14: pBR322 DNA cleaved by Eu^{3+}, Gd^{3+}, La^{3+}, Nd^{3+} and Pr^{3+} (2×); 20 h/50 °C.

4. Conclusions

Sterically or electrostatically stabilized colloidal magnetite was obtained by precipitation of Fe^{2+} and Fe^{3+} salts and encapsulated by dispersion (co)polymerization of HEMA and GMA. Properties (morphology, size and its distribution, iron content) of prepared polymer microparticles were controlled by subtle changes in various reaction parameters, such as polarity of the reaction mixture, stabilizer and its concentration, initiator and monomer, polymerization temperature. Several enzymes, such as RNase A, DNase I, anti-*Salmonella* and proteinase K, were immobilized on the microparticles and their applicability in degradation of bacterial RNA, chromosomal and plasmid DNA, magnetic separation of *Salmonella* cells or degradation of their intracellular inhibitors was demonstrated. The effect of several components used in the microparticle preparation on PCR was investigated on DNA isolated from G^+ bacteria *Bifidobacterium longum*. Magnetite nanoparticles, Fe^{3+} ions, oleic acid or PEG partially inhibited PCR or gave falsely negative results, similarly to P(HEMA-*co*-EDMA) microparticles containing needle-like maghemite or oleic acid-coated magnetite. Surprisingly, magnetic PGMA particles encapsulating electrostatically stabilized colloidal magnetite did not interfere with PCR suggesting complete magnetite incorporation inside the PGMA microspheres. Genomic DNA was successfully isolated from cell lysate on weakly acid derivatives of magnetic P(HEMA-*co*-EDMA) and P(HEMA-*co*-GMA) microparticles in the presence of PEG and sodium chloride. Finally, heterogeneous lanthanide complexes (Eu^{3+}, Gd^{3+}, La^{3+}, Nd^{3+} and Pr^{3+}) of ethylenediaminetetraacetic acid immobilized on insoluble PGMA support were used as catalysts of pBR322 DNA cleavage.

Acknowledgements

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