Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 800 (2004) 27–32
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Magnetic hydrophilic methacrylate-based polymer microspheres designed for polymerase chain reactions applications

Alena Španová[a], Daniel Horák[b], Eva Soudková[a], Bohuslav Rittich[a,*]

[a] *Department of Microbiology, Faculty of Science, Masaryk University Brno, Tvrdého 14, 602 00 Brno, Czech Republic*
[b] *Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského Sq. 2, 162 00 Prague 6, Czech Republic*

Abstract

Magnetic hydrophilic non-porous P(HEMA-*co*-EDMA), P(HEMA-*co*-GMA) and PGMA microspheres were prepared by dispersion (co)polymerization of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA) or glycidyl methacrylate (GMA) in the presence of several kinds of magnetite. It was found that some components used in the preparation of magnetic carriers interfered with polymerase chain reaction (PCR). Influence of non-magnetic and magnetic microspheres, including magnetite nanoparticles and various components used in their synthesis, on the PCR course was thus investigated. DNA isolated from bacterial cells of *Bifidobacterium longum* was used in PCR evaluation of non-interfering magnetic microspheres. The method enabled verification of the incorporation of magnetite nanoparticles in the particular methacrylate-based polymer microspheres and evaluation of suitability of their application in PCR. Preferably, electrostatically stabilized colloidal magnetite (ferrofluid) should be used in the design of new magnetic methacrylate-based microspheres by dispersion polymerization.

Keywords: Polymerase chain reaction inhibition; Magnetic microspheres; Methacrylates

1. Introduction

Proteins and enzymes immobilized on solid supports find applications in different diagnostic procedures [1–5]. Separation and identification of microbial species is a fundamental microbiological technique. In medical and food microbiology, specific microorganisms must be detectable in the presence of dominant background microflora and in heterogenous media.

The occurrence of falsely negative results is a problem in the routine testing of various real samples. When polymerase chain reaction (PCR) is used for the identification of target cells, falsely negative results can be caused by the presence of intracellular or extracellular inhibitors [6–8]. The above-mentioned problem can be solved using immunomagnetic separation (IMS) of cells. Magnetic microbeads carrying specific antibodies are used for capturing and separation of target cells from the environment containing extracellular inhibitors or competitive microflora [5–7]. They need not to be detached from the target microbial cells and the IMS technique can be effectively combined with other methods of microbial cell identification, such as cultivation (IMS-CM) or PCR (IMS-PCR). However, the microbeads used must not interfere with the course of PCR.

Commercially available styrene-divinylbenzene microbeads, which possess hydrophobic properties, are currently used for immobilization of cells and proteins [9]. Non-specific adsorption of DNA released from heated non-viable cells can be the cause of false positive results [5]. Attention is therefore given to microspheres with hydrophilic properties, the advantage of which consists in low non-specific adsorption of biologically active compounds. Magnetic poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) (P(HEMA-*co*-EDMA)), microspheres were prepared by dispersion polymerization and used for the attachment of polyclonal *Salmonella* antibodies [5]. However, sensitivity of the target cell determination using IMS-PCR was lower compared with commercial magnetic Dynabeads anti-Salmonella beads.

The aim of this study was to prepare magnetic microspheres with hydrophilic properties, such as exemplified by P(HEMA-*co*-EDMA), poly(2-hydroxyethyl methacrylate-

* Corresponding author.
E-mail address: rittich@sci.muni.cz (B. Rittich).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.09.010

co-glycidyl methacrylate) (P(HEMA-*co*-GMA)) and poly-(glycidyl methacrylate) (PGMA), that do not interfere with polymerase chain reaction. Influence of microspheres on the PCR course, including various components used in their preparation, was therefore investigated.

2. Materials and methods

2.1. Chemicals

Agarose was purchased from Lachema (Brno, Czech Republic); ethidium bromide from Sigma (St. Louis, USA). Cubic magnetite (ferrous–ferric oxide Fe_3O_4, 200 nm) was prepared in the Institute of Inorganic Chemistry, Academy of Sciences of the Czech Republic (Řež, Czech Republic), colloidal magnetite (ferrofluid) by coprecipitation of Fe^{2+} and Fe^{3+} salts in alkaline medium at 90 °C [10]. Monomers, 2-hydroxyethyl methacrylate (HEMA), Röhm (Darmstadt, Germany), ethylene dimethacrylate (EDMA), Aldrich (Milwaukee, USA), and glycidyl methacrylate (GMA), Fluka (Buchs, Switzerland), were purified by distillation under reduced pressure in nitrogen atmosphere. Cellulose acetate butyrate was a kind gift of Eastman (Kingsport, USA). Primers PbiF1 and PbiR2 [11] were synthesized by Generi-Biotech (Hradec Králové, Czech Republic), Rec Taq polymerase was from Top-Bio (Prague, Czech Republic) and DNA marker pBR322/BstNI (1857, 1058, 929, 383, and 121 bp) used in gel electrophoresis was from New Englands BioLabs (Hertfordshire, UK). Oleic acid, poly(ethylene glycol) (PEG, M_w = 6000), poly(vinylpyrrolidone) (PVP K 15, M_w = 10 000), were from Fluka (Buchs, Switzerland). The other chemicals were of analytical grade and from commercial sources.

2.2. Equipment

Magnetic microspheres were separated using an MPC-M magnetic particle concentrator, Dynal (Oslo, Norway). The reaction mixture was amplified on MJ Research Programme Cycler PTC-100 (Watertown, USA). The agarose gel electrophoresis apparatus was from Bio-Rad Labs (Richmond, USA). The PCR products were visualized on UV transluminator EB-20E from UltraLum (Paramount, USA), and photographed with CD 34 Polaroid Camera (Cambridge, USA). Spectrophotometric measurements were carried out on UV-Vis spectrophotometer DMS 100S Varian Techtron (Mulgrave, Australia).

2.3. Microorganisms

Bacterial cells of *Bifidobacterium longum* (Laktoflora, Prague, Czech Republic) were used for DNA isolation. The *Bifidobacterium* cells were chosen due to their safety during manipulation.

2.4. Methods

2.4.1. Preparation of carriers

Magnetic non-porous P(HEMA-*co*-EDMA) (92/8, w/w) microspheres were prepared by cellulose acetate butyrate-stabilized and dibenzoyl peroxide-initiated dispersion copolymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate in a toluene/2-methylpropan-1-ol medium in the presence of cubic magnetite according to the previously described procedure [12]. Magnetic P(HEMA-*co*-GMA) (1/1, w/w) microspheres were obtained in the same medium and under analogous conditions in the presence of colloidal oleic acid-coated magnetite particles (ferrofluid). Poly(ethylene glycol)—PEG 6000 and poly(vinylpyrrolidone)—PVP K 15, were used for coating of colloidal magnetite particles, too. Magnetic PGMA microspheres were prepared by poly(vinylpyrrolidone)-stabilized and 2,2′-azobisisobutyronitrile-initiated dispersion polymerization of GMA in ethanol in the presence of colloidal magnetite treated with either perchloric acid or tetramethylammonium hydroxide (TMAH). In all cases, polymerizations were run at 70 °C for 16 h. Some properties of the synthesized microspheres are summarized in Table 1.

2.4.2. Cell cultivation and DNA isolation

Bacterial cells of *B. longum* were cultivated anaerobically on MRS medium (Oxoid) with cysteine (0.5 g/l) overnight (18 h). Altogether 1 ml of cells was washed and resuspended in 100 μl of lysis buffer (10 mM Tris–HCl, pH 7.8, 5 mM EDTA, pH 8.0; lysozyme 0.3 mg/ml); 10 μl of proteinase K (10 mg/ml) and 2.5 μl of SDS (20%) were then added and the mixture was incubated for 18 h at 55 °C. The crude cell lysates were used for phenol extraction of DNA [13]. Identity of nucleic acids was checked by gel electrophoresis and UV spectrophotometry. The ratio $A_{260\,nm}/A_{280\,nm}$ was used as a test of nucleic acid purity [14].

Table 1
Characteristics of prepared magnetic microspheres

Polymer	Encapsulated Fe_3O_4	Fe content (wt.%)	Diameter (μm)	PDI
P(HEMA-*co*-EDMA) (92/8, w/w)	Cubic (200 nm)	8.1	1.18	1.07
P(HEMA-*co*-GMA) (1/1, w/w)	Oleic acid-coated, colloid	5.3	1.71	1.05
PGMA	$HClO_4$-treated, colloid	12.9	0.36	1.04
PGMA	$(CH_3)_4NOH$-treated, colloid	5.9	0.74	1.07

PDI: polydispersity index (the weight-to-number average diameter of the particles).

Table 2
Effect of non-magnetic P(HEMA-*co*-EDMA), magnetic P(HEMA-*co*-EDMA) and PGMA microspheres on PCR course

Sample	Microsphere/PCR mixture (μg/25 μl)	*Bifidobacterium bifidum* DNA/PCR mixture (pg/25 μl)/PCR product			
		2000	200	20	2
Control	0	++	++	+	+/−, −
Non-magnetic P(HEMA-*co*-EDMA)	5	++	+	+	+/−
Non-magnetic PGMA	5	+++	++	+	+/−
Magnetic P(HEMA-*co*-EDMA)[a]	5	−*	−*	−*	−*
	5×10^{-2}	++	+	+	−
Magnetic P(HEMA-*co*-GMA)[b]	5	+++	++	+	+/−
Magnetic PGMA[c]	5	+++	++	+	+/−
Magnetic PGMA[d]	5	+++	++	+	+/−

PCR product: (+++), (++), (+), (+/−) band of very strong, strong, mean and weak intensity; (−) no band; (*) false negative result.
[a] Cubic Fe_3O_4 encapsulated in the microspheres.
[b] Oleic acid-coated Fe_3O_4 encapsulated in the microspheres.
[c] Perchloric acid-treated Fe_3O_4 encapsulated in the microspheres.
[d] Tetramethylammonium hydroxide-treated Fe_3O_4 encapsulated in the microspheres.

Table 3
The effect of components on PCR course

Component	Component/PCR mixture (μg/25 μl)	Magnetic separation	*Bifidobacterium bifidum* DNA/PCR mixture (pg/25 μl)/PCR product			
			2000	200	20	2
Control	0	−	+++	++	++	+/−, −
Cubic Fe_3O_4	5	−	+++	+	+/−	−
	5×10^{-2}	−	+++	+++	++	−
	5	+	+++	+++	+	+/−
Oleic acid	5	−	+++	+/−	+/−	−
	5×10^{-2}	−	+++	++	+	+/−
PVP K 15	5	−	+++	++	+	+/−
PEG 6000	5	−	+++	−**	−**	−**
	5×10^{-2}	−	+++	++	+	−
TMAH	5	−	+++	++	++	+
$HClO_4$	5×10^{-3}	−	−**	−**	−**	−**
Fe_3O_4 (oleic acid*)	5	−	+++	−**	−**	−**
Fe_3O_4 (PVP K 15*)	5	−	−**	−**	−**	−**
	5×10^{-2}	−	++	+	+/−	−
	5	+	+	−**	−**	−**
	5×10^{-2}	+	+++	++	+	−
Fe_3O_4 (PEG 6000*)	5	−	−**	−**	−**	−**
	5×10^{-2}	−	++	+	+/−	−
	5	+	−**	−**	−**	−**
	5×10^{-2}	+	+++	++	+	+/−

PCR product: (+++), (++), (+), (+/−) band of very strong, strong, mean and weak intensity; (−) no band; (*) coating; (**) false negative result; TMAH: tetramethylammonium hydroxide.

2.4.3. PCR amplification and detection of PCR products

Purified DNA (by phenol extraction method) served as DNA matrix in PCR. PCR was performed using PbiF1 and PbiR2 primers which enabled to amplify a 914 bp long DNA fragment specific to the *Bifidobacterium* genus [11]. Typically, the PCR mixture contained 0.5 μl each 10 mM dNTP, 0.5 μl (10 pmol/μl) each primer, 1 μl DNA matrix, 0.5 μl RecTaq polymerase 1.1 (1 U/μl), 2.5 μl buffer; PCR water was then 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 at 305 nm UV light on TT667 film. The lengths of

amplified DNA fragments were calculated using the Anagel programme [15].

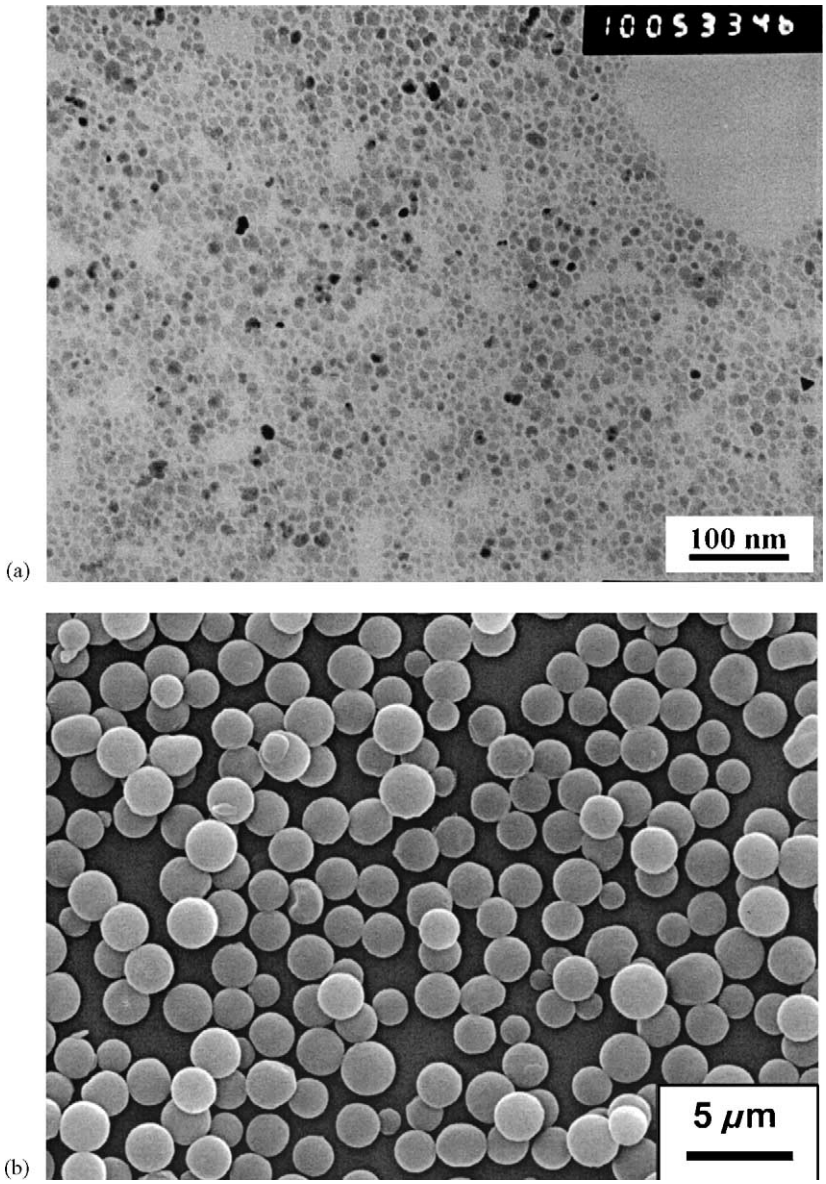
2.4.4. Influence of microspheres and components on the PCR course

Influence of microspheres or components (used in preparation of magnetic microspheres) on the PCR course was studied according to the following procedure. Microspheres or individual components (10 mg) were mixed with 1 ml of sterile distilled water, 10 μl of suspension (emulsion) was added to 10 μl DNA, mixed, and 1 μl of the mixture was used in PCR. Supernatants were obtained by magnetic separation, if magnetic microspheres were investigated. Altogether four different DNA amounts (2 ng, 200, 20, and 2 pg/25 μl of PCR mixture) were used for PCR amplification.

3. Results and discussion

In a previous work [5], magnetic P(HEMA-*co*-EDMA) microspheres prepared by dispersion polymerization were used for the attachment of polyclonal *Salmonella* antibodies. Presence of interfering components decreased PCR sensitivity (only higher amounts of DNA gave detectable PCR products), or led to false negative results. Consequently, sensitivity of target cell determination using PCR was lower in comparison with commercially available magnetic Dynabeads anti-Salmonella beads.

In this report, effect of various microspheres, including magnetic ones or individual components used in their preparation, on PCR sensitivity was tested in detail using DNA isolated from *B. longum* cells. First, the effect of



(a)

(b)

Fig. 1. Transmission (a) and scanning electron micrograph (b) of toluene-sprayed oleic acid-coated magnetite (a) and magnetic P(HEMA-*co*-GMA) microspheres (b) prepared in its presence.

non-magnetic P(HEMA-*co*-EDMA) and PGMA particles on the PCR course was studied. It was shown that the PCR course was not influenced by their presence (Table 2), i.e. no particles or components used in their preparation did interfere with PCR.

Attention was then focused on the study of PCR inhibition by cubic or colloidal magnetites (a core material in magnetic methacrylate-based polymer microspheres) and compounds used for their stabilization (Table 3). Different amounts of them were tested in polymerase chain reaction as dilution of sample represents a simple method that can facilitate amplification in the presence of inhibitors. The use of different DNA amounts enables to determine the differences in PCR sensitivity. And indeed, all magnetite particles partially inhibited the PCR course or give false negative results. Polymerase chain reaction was probably influenced by the presence of ferric ions in the solution. This assumption was experimentally confirmed by using different amount of Fe^{3+} ions in PCR mixture (0.1×10^{-12} to 1×10^{-12} mM). The course of PCR was inhibited by Fe^{3+} ions at a concentration 0.1 μM and higher. Equilibrium between Fe^{3+} ions in magnetic nanoparticles and the solution is namely dynamic, i.e., Fe^{3+} ions can cross from the solid phase to the solution and vice versa. The assumption was further proved by only a moderate PCR inhibition by a supernatant remaining after removal of PEG- or PVP-coated magnetite nanoparticles using magnetic separator. In addition to magnetite, the PCR course was inhibited also by other components used in the nanoparticle synthesis, especially by PEG (Table 3).

Magnetic P(HEMA-*co*-EDMA) microspheres are known to aggregate in water solutions, which could reduce the steric accessibility of immobilized ligands (proteins) and disqualify their medico-biological application. To obviate this shortcoming, a new design of magnetic P(HEMA-*co*-GMA) microspheres preparation was suggested. Colloidal magnetite obtained by chemical coprecipitation of Fe^{2+} and Fe^{3+} salts in ammonia and incorporated into the microspheres during the polymerization was first coated with several compounds, such as oleic acid, poly(ethylene glycol) and poly(vinylpyrrolidone), to prevent a coalescence [10]. The compounds stabilized magnetite sterically. Moreover, they influenced the quality of resulting magnetic microspheres, e.g., morphology, size and polydispersity (Table 1); coating of colloidal magnetite with oleic acid appeared to be the most suitable (Table 2). Oleic acid-coated magnetite nanoparticles, mostly in the 10 ± 5 nm diameter range, are shown in Fig. 1a. Their good dispersibility in non-polar solvents is a result of their hydrophobization. Magnetic P(HEMA-*co*-GMA) microspheres of 1.7 μm diameter prepared by dispersion polymerization in toluene/2-methylpropan-1-ol medium in the presence of oleic acid-coated magnetite (ferrofluid) are shown in Fig. 1b. The magnetic microspheres prepared in this way can be applied in immobilization techniques (Fig. 2). Colloidal magnetite coated with PEG 6000 or PVP K 15 had a tendency to agglomerate and was apparently not completely encapsulated in the polymer microspheres. It can be thus speculated that PEG 6000 or PVP K 15 coated magnetite preferentially covered the microspheres surface and therefore interfered with the PCR course. It can be noted that coating of magnetite with PVP K 15 or PEG 6000 before its encapsulation by dispersion polymerization affected the sensitivity of PCR approximately in the same way as magnetic P(HEMA-*co*-EDMA) microspheres containing cubic magnetite (Tables 2 and 3).

It follows from the above mentioned that it is necessary to pay attention to complete encapsulation of magnetite in polymer microspheres. Therefore, electrostatically stabilized colloidal magnetite was developed and encapsulated by dispersion polymerization of GMA in ethanol resulting in magnetic PGMA microspheres (Table 1). It is just to remind that while electrostatically stabilized magnetite forms ferrofluid in ethanol, oleic acid-coated magnetite nanoparticles coagulate in this medium. The advantage of PGMA microspheres consists in that they do not have a 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. Electrostatic stabilization of magnetite in water/ethanol was achieved by its treatment with either perchloric acid or tetramethylammonium hydroxide and the resulting ferrofluid nanoparticles did not substantially differ in size from that of the oleic acid-coated ones in toluene. And indeed, magnetic PGMA microspheres prepared by this technique did not aggregate in water solutions nor detectably interfered with PCR (Table 2, Fig. 2) even if perchloric acid interfered with PCR course (Table 3). It means that colloidal magnetite was predominantly incorporated inside the PGMA microspheres.

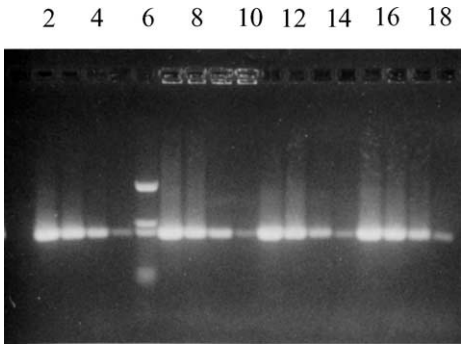


Fig. 2. Agarose gel electrophoresis of PCR products obtained after amplification of *Bifidobacterium bifidum* DNA in the presence of magnetic P(HEMA-*co*-GMA) microspheres and PGMA microspheres (without magnetic separation) containing magnetite treated in different ways before encapsulation. Lane 1: negative controls without DNA; lanes 2–5: controls with 2 ng, 200, 20 and 2 pg of DNA/25 μl of PCR mixture; lane 6: DNA standard pBR322/BstNI; lanes 7–10, 11–14 and 15–18: DNA amplified in the presence of P(HEMA-*co*-GMA, 1/1), oleic acid-coated Fe_3O_4 encapsulated in the microspheres; PGMA: perchloric acid-treated Fe_3O_4 encapsulated in the microspheres; PGMA: tetramethylammonium hydroxide-treated Fe_3O_4 encapsulated in the microspheres, respectively.

4. Conclusion

Knowledge of interference of cubic and colloidal magnetite with the course of polymerase chain reaction helped in the design of new magnetic hydrophilic methacrylate-based polymer microspheres. The method enables non-direct verification of incorporation of magnetite nanoparticles in the microspheres and evaluation of applicability of individual magnetic carriers in PCR. Preferably, electrostatically stabilized colloidal magnetite (ferrofluid) was used in the design of new magnetic PGMA microspheres by dispersion polymerization in ethanol. The advantage of these microspheres is their easy dispersibility in water. Oleic acid-coated colloidal magnetite (ferrofluid) can be then recommended for the preparation of magnetic P(HEMA-*co*-GMA) microspheres in toluene/2-methylpropan-1-ol medium.

Acknowledgements

Supported by the Grant Agency of the Czech Republic, grant nos. 202/02/1361 and 525/02/0287, and the National Grant Agency for Agricultural Research (NAZV), grant no. QD 1377.

References

[1] B. Sinclair, Scientist 12 (1998) 17.
[2] J. Turková, J. Chromatogr. B 722 (1999) 11.
[3] I. Šafařík, M. Šafaříková, J. Chromatogr. B 722 (1999) 33.
[4] F. Jelen, B. Yosypchuk, A. Kouřilová, L. Novotný, E. Paleček, Anal. Chem. 74 (2002) 4788.
[5] A. Španová, B. Rittich, D. Horák, J. Lenfeld, J. Prodělalová, J. Sučiková, S. Štrumcová, J. Chromatogr. A 1009 (2003) 215.
[6] N. Rijpens, L. Herman, F. Verecken, G. Jannes, J. DeSmedt, L. DeZutter, Int. J. Food Microbiol. 46 (1999) 37.
[7] A. Španová, B. Rittich, R. Karpíšková, L. Čechová, D. Škapová, Bioseparation 9 (2001) 379.
[8] G. Wilson, Appl. Environ. Microbiol. 63 (1997) 3741.
[9] Anonymous, Cell Separation and Protein Purification, second ed., Dynal, Oslo, Norway, 1996.
[10] D. Horák, N. Semenyuk, F. Lednický, J. Polym. Sci., Polym. Chem. Ed. 41 (2003) 1848.
[11] D. Roy, S. Sirois, FEMS Microbiol. Lett. 191 (2000) 17.
[12] D. Horák, B. Rittich, J. Šafář, A. Španová, J. Lenfeld, M.J. Beneš, Biotechnol. Prog. 17 (2001) 447.
[13] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, second ed., Cold Spring Harbor Laboratory Press, New York, 1989, pp. B20–B21.
[14] R.R. Sinden, DNA Structure and Function, Academic Press, San Diego, 1994, p. 34.
[15] J. Mrázek, A. Španová, CABIOS 8 (1992) 524.