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Immunomagnetic separation and detection of *Salmonella* cells using newly designed carriers

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Abstract

Magnetic nonporous poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) microspheres were prepared by dispersion copolymerisation of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA) or glycidyl methacrylate (GMA) in the presence of magnetite. They were functionalized by polyclonal *Salmonella* antibodies via the trichlorotriazine method. *Salmonella* cells were then successfully identified using cultural and polymerase chain reaction (PCR) methods after their immunomagnetic separation. The PCR sensitivity of target cell detection was negatively influenced by the presence of some compounds used in the process of particle preparation. In some cases, magnetic poly(HEMA–*co*-EDMA) microspheres with immobilized proteinase K were used for degradation of intracellular inhibitors present in *Salmonella* cells.

Keywords: *Salmonella* spp.; Magnetic sorbents; Immobilized proteins; Affinity sorbents; Hydroxyethyl methacrylate; Glycidyl methacrylate; Ethylene methacrylate; Proteinase K; Enzymes; Antibodies

1. Introduction

Proteins and enzymes immobilized on solid supports find application in biosciences and special diagnostic procedures [1–5]. The 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.

The incidence of infections caused by pathogenic *Salmonella* bacteria present in foods has significantly increased in many countries during the last two decades [6,7]. Conventional cultivation methods (CMs) [8,9] and molecular diagnostic techniques, such as polymerase chain reaction (PCR), have been used for the identification of *Salmonella* cells [10–12]. The occurrence of falsely negative results is a problem in the routine testing of various foodstuffs on the market or clinically suspect samples. If PCR has been used for the identification of target cells, falsely negative results can be caused by the presence of intracellular or extracellular inhibitors [11–13]. The above mentioned problem can be solved using immunomagnetic separation (IMS) of cells. Magnetic particles carrying specific antibody can be used for the capture of target cells and their separation from the environment containing extracellular inhibitors or competitive microflora [5,11,12]. Mag-

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netic particles need not be detached from the target microbial cells, and the IMS technique can be effectively combined with other methods of microbial cell identification. As we already showed [12], IMS-PCR saves time necessary for confirmation of *Salmonella* cells including strains with unusual biochemical reactions.

Commercially available Dynabeads anti-*Salmonella* magnetic particles (Dynal, Oslo, Norway) are suitable for the separation of *Salmonella* cells. As Dynabeads particles are characterized by hydrophobic properties, magnetic hydrophilic nonporous microspheres, 1.2 μm in size, were developed. They were prepared by dispersion copolymerization of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA) or glycidyl methacrylate (GMA). Poly(HEMA-*co*-EDMA) is known to be a highly hydrophilic and biocompatible hydrogel with a low nonspecific protein adsorption and a good chemical stability. It imbibes large amounts of water, and its hydroxy groups can be easily modified [14]. It has been also used as a support in chromatographic applications [15]. Previously, we used magnetic nonporous poly(HEMA-*co*-EDMA) microspheres with immobilized RNase A and DNase I for digestion of high-molecular-mass RNA [14], chromosomal DNA and specific cleavage of plasmid DNA [16]. The magnetic nonporous poly(HEMA–*co*-EDMA) microspheres were also used as a support in the enzyme reactor with immobilized galactose oxidase and neuramidase [17].

The aim of this work was to test our newly designed magnetic nonporous poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) microspheres functionalized by polyclonal *Salmonella* antibodies via the trichlorotriazine method for immunomagnetic separation of *Salmonella* cells and their identification in IMS-CM and/or IMS-PCR. The second aim was PCR identification of *Salmonella* cells containing intracellular PCR inhibitors after sample pre-treatment by immobilized proteinase K.

2. Materials and methods

2.1. Chemicals

Agarose was purchased from Lachema (Brno, Czech Republic); ethidium bromide, 2,4,6-trichloro-1,3,5-triazine (TCT, cyanuric chloride), and proteinase K from Sigma (St. Louis, MO, 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). Specific polyclonal *Salmonella* antibody was a kind gift of Dr. E. Brynda (Institute of Macromolecular Chemistry, Prague, Czech Republic). Monomers, HEMA (Röhm, Darmstadt, Germany), EDMA (Aldrich, Milwaukee, WI, USA), and GMA (Fluka, Buchs, Switzerland) were purified by distillation under reduced pressure in a nitrogen atmosphere. Cellulose acetate butyrate (CAB) was a kind gift of Eastman (Kingsport, USA). Primers ST11 and ST15 [10] were synthesized by Generi-Biotech (Hradec Králové, Czech Republic), RecTaq polymerase and DNA markers 970-155 (used in gel electrophoresis) were purchased from Top-Bio (Prague, Czech Republic), Dynabeads anti-*Salmonella* magnetic particles were from Dynal. The other chemicals were of analytical grade and from commercial sources.

2.2. Equipment

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

2.3. Microorganisms and samples

Bacterial cells of *Salmonella enterica* ser. Typhimurium LB 5000 and *S. enterica* ser. Typhimurium LT2-18 were used for the evaluation of immunomagnetic separation. The field bacterial strain *S. arizonae* 18/78 with a high content of intracellular PCR inhibitors was used in this study. It

was isolated from a stool sample of a human patient [9].

2.4. Methods

2.4.1. Preparation of carriers

Magnetic poly(HEMA-*co*-EDMA) and poly-(HEMA-*co*-GMA) microspheres were prepared by cellulose acetate butyrate-stabilized and dibenzoyl peroxide (BPO)-initiated dispersion copolymerization of HEMA and EDMA or GMA in a toluene-2-methylpropan-1-ol medium in the presence of magnetite according to a previously described procedure [14]. The size of microspheres was 1.2 μm with a polydispersity index (the mass-to-number average diameter of the particles) of 1.07.

2.4.2. Immobilization technique

To attach *Salmonella* antibodies and proteinase K, magnetic poly(HEMA-*co*-EDMA) and hydrolyzed (0.05 *M* sulfuric acid, 5 h/50 °C) poly(HEMA-*co*-GMA) microspheres were activated with 2,4,6-trichloro-1,2,3-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 [18]. The immobilization itself proceeded for 4 h at 23 °C and pH 8. The product was repeatedly washed with water and lyophilized.

Magnetic poly(HEMA-*co*-EDMA) microspheres contained 3.1 mg antibody/g or 10.2 mg proteinase K/g of carrier, and magnetic poly(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.

2.4.3. Cell cultivation and sample pre-treatment

Bacterial cells of *S. enterica* Typhimurium LB 5000 and *S. enterica* Typhimurium LT2-18 were grown overnight (18 h) on LB agar plates. The cultures were heated for 10–60 min at 48, 72 and 100 °C, if the temperature effect of cell pre-treatment on IMS efficiency was studied. Temperatures of 4 and 24 °C and incubation times of 5–30 min were tested for the optimization of IMS conditions using magnetic poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) microspheres. The cells treated were separated by anti-*Salmonella* particles according to the procedure given in Section 2.4.4. Altogether 25 μl of bacterial cells or bacterial cell–immunomagnetic particle complexes was dropped on LB agar plates. The plates were incubated for 48–72 h at 37 °C. Each experiment was repeated 3–6 times.

2.4.4. Immunomagnetic separation

The separation of target cells was carried out according to slightly modified instructions published by the producer [19]. Briefly, a total of 20 μl of Dynabeads anti-*Salmonella* magnetic particles was added to 1 ml of culture grown in liquid LB medium. The suspension was incubated for 10 min at room temperature under gentle agitation. In the case of magnetic poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) anti-*Salmonella* microspheres, 10 μl of particles was used under the tested conditions. Magnetic particles with the attached cells were isolated using a magnetic separator, pre-washed with 1 ml of phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20, and separated using a magnetic separator (twice). The supernatant was removed and magnetic particles were resuspended in 100 μl of PCR water. The *Salmonella* cells attached to immunomagnetic particles were cultivated on agar plates or used directly in PCR.

2.4.5. PCR amplification and detection of PCR products

Both purified DNA (phenol extraction method) [20] and DNA from cell lysates were used as DNA matrix in PCR. PCR was performed using ST11 and ST15 primers which enabled amplification of a 429 base pair (bp) long DNA fragment specific to the *Salmonella* genus [10]. Typically, the PCR mixture contained 1.5 μl of 25 m*M* $MgCl_2$, 1 μl of each 10 m*M* deoxyribose nucleotide triphosphate (dNTP), 0.5 μl (10 pmol/μl) of each primer, 2 μl of DNA matrix, 1 μl of RecTaq polymerase (0.5 U/μl), 2.5 μl of Rec Taq buffer and PCR water was added to a 25 μl volume. After 5 min of the initial denaturation period at 94 °C (hot start), amplification was carried out in 35 cycles of 40 s at 94 °C, 40 s at 57 °C, and 80 s at 72 °C. In the last cycle, the elongation step at 72 °C was prolonged to 10 min. The PCR products were detected by agarose gel

electrophoresis in 1.8% agarose gel in TBE buffer (45 m*M* boric acid, 45 m*M* Tris base, 1 m*M* EDTA, pH 8.0) after ethidium bromide staining (0.5 μg/ml), decolourized in water, and photographed at 305 nm UV light on a TT667 film. The DNA from the bacterial strain *Escherichia coli* JM 109 was used as a negative control, purified DNA from *Salmonella* Typhimurium LB 5000 served as a positive control. The lengths of amplified DNA fragments were calculated using the Anagel programme [21].

3. Results and discussion

3.1. Immunomagnetic separation of Salmonella cells

A higher number of false negative results was obtained using the conventional CM or PCR without IMS compared with IMS-CM [9] or IMS-PCR [12], respectively, when dealing with the identification of *Salmonella* cells in dried food samples. This was apparently caused by the fact that *Salmonella* cells present in processed foods were not under the same physiological conditions as they were in pure laboratory cultures. *Salmonella* cells present in processed foods (especially dried foods, such as milk powder and eggs) can be sublethally damaged during drying. In the process of pre-enrichment, the growth of *Salmonella* cells can be suppressed by competitive microflora. As a result, they may not be detected using the conventional cultivation methods [9].

As *Salmonella* cells are temperature-sensitive, the influence of cell heating on the possibility of their identification after IMS was studied. We carried out cell separation using Dynabeads anti-*Salmonella* magnetic particles (followed by PCR identification) from a suspension of *S. enterica* Typhimurium LB5000 cells which were heated at 48, 72 and 100 °C for 10–60 min. In the case of identification of target cells using CM (without IMS) and IMS-CM, no bacterial growth was detected as early as after 10 min of heating at 72 and 100 °C. *Salmonella* cells are known to survive only for several minutes at 70 °C in different media [22]. On the contrary, specific PCR products were identified at these temperatures using IMS-PCR (see Fig. 1). In comparison with simple PCR (without IMS), the intensity of

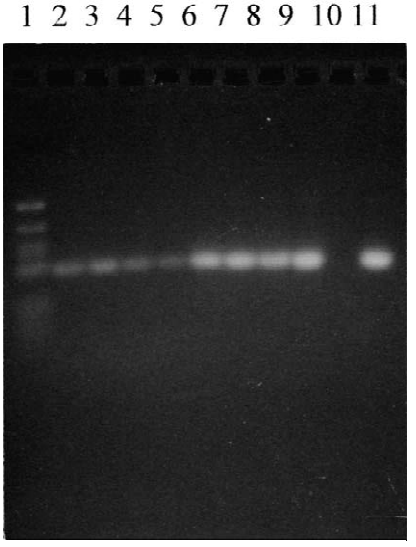

Fig. 1. Agarose gel electrophoresis of IMS-PCR products of heated *S. enterica* Typhimurium LB 5000 cells. Conditions: 0.8% agarose gel, TBE (45 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0) buffer. Lanes: 1=DNA standards (155–970 bp); 2–5=cells were heated at 100 °C for 10, 20 and 30 min, followed by IMS; 6–9=cells were heated at 100 °C for 10, 20 and 30 min, without IMS; 10=control without DNA; 11=control DNA *S. enterica* Typhimurium LB 5000.

PCR products obtained after IMS-PCR was lower. This can be explained either by the capture of a small number of dead cells on immunomagnetic particles or by the capture of a low amount of DNA matrix in the sample mixture which was liberated from ruptured cells. As PCR is a very sensitive method, the target DNA sequence from dead cells can be amplified in a sufficient amount by PCR. It can be only speculated on the character of the incidental cell sorption. It may occur by the interaction of an antibody with non-destroyed epitope on the cell surface or by the physicochemical sorption on the particle surface (even if the particles were washed with distilled water after IMS, this washing could be insufficient).

Therefore, the newly designed hydrophilic particles were tested for immunomagnetic separation of *Salmonella* cells. The advantage of hydrophilic microspheres consists in a low non-specific adsorption of biologically active compounds. Magnetic poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA)

particles were prepared by dispersion polymerization (see Section 2.4.1), which is a suitable technique for obtaining spherical particles of relatively narrow distribution in a micrometer size range. The critical condition for this technique is that the monomers to be polymerized, the stabilizer and the initiator must be dissolved to a homogeneous solution in a solvent mixture, whereas the arising polymer must be insoluble. A cellulose acetate butyrate stabilizer has to be used to prevent particle agglomeration. The solvency of the medium determines the critical molecular mass, above which the polymer precipitates on magnetite, and, ultimately, the final particle size. First, nuclei are formed, which are associated in spherical particles reaching a size in the range of micrometers and with a narrow size distribution. The particle size of both poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) was reduced with the decreasing solvency of the reaction medium (more nuclei were generated) because the critical chain length of the precipitated oligomers decreased with an increasing toluene content, which is a poorer solvent for the polymer than 2-methylpropan-1-ol. There is an optimum initiator concentration (2%, w/w BPO relative to monomers) for producing low-polydispersity particles under given conditions (Fig. 2).

Prepared magnetic nonporous poly(HEMA-*co*-EDMA) and poly(HEMA-*co*-GMA) microspheres were used for the attachment of polyclonal *Salmonella* antibodies and subsequently in the next series of batch experiments. The operational suitability of our newly designed particles was proved by immunomagnetic separation of *Salmonella* cells followed by cultivation and PCR identification.

First, the best conditions for the attachment of cells on immunomagnetic particles were searched for (see Section 2.4.3). The number of attached cells was estimated using the cultivation method. Even though the number of attached cells was appropriate (as several cells attached on a particle or its aggregate can give only one colony), this procedure was used for verification of the functionality of immobilised antibodies. The results are given in Table 1. The higher variability in the number of attached target cells in the case of poly(HEMA-*co*-EDMA), contrary to poly(HEMA-*co*-GMA) microspheres, was apparently caused by the particle agglomeration in the environment of the buffer used (aqueous medium), which resulted in a poorer availability of the immobilized antibody. This fact was confirmed by microscopic observation. The amount of attached cells proportionally increased with the amount of the used immunomagnetic poly(HEMA-*co*-EDMA) particles (results not shown). From tested separation conditions were used: temperature 24 °C and 15 min of separation both for poly(HEMA-*co*-EDMA) and

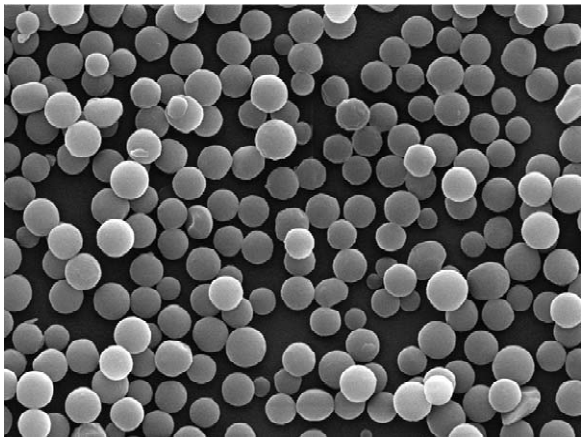

Fig. 2. Scanning electron micrograph of magnetic poly(HEMA–*co*-GMA) microspheres obtained by dispersion polymerization.

Table 1
The effect of experimental conditions on immunomagnetic separation of *Salmonella* Typhimurium cells

Particle	Time (min)	Strain/temperature (°C), average number of attached cells			
		LT2-18		LB-5000	
		4	24	4	24
A	5	180	170	130	230
	15	250	240	250	250
	30	250	250	210	250
B	5	650	470	930	850
	15	160	400	240	620
	30	660	650	1510	1480

The number of cells (attached to 10 μl of particles used) was determined by the cultivation method. Experimental conditions were described in Materials and methods.

A—Magnetic poly(HEMA-*co*-EDMA) microspheres, B—magnetic poly(HEMA-*co*-GMA) microspheres.

30 min for poly(HEMA-*co*-GMA) microspheres. The producer of Dynabeads anti-*Salmonella* magnetic particles recommends cell incubation for 10 min at laboratory temperature.

Newly designed particles were also used in IMS-PCR. The sensitivity of determination of the PCR product was lower in comparison with magnetic Dynabeads anti-*Salmonella* particles. Sensitivity of the detection was evidently negatively influenced by the presence of some compounds used in the process of particle preparation. The influence of naked particles and individual compounds used during the preparation of magnetic carriers on the course of PCR was therefore tested (results are not shown). Purified DNA isolated from bacterial strains was mixed with different amounts of particle suspension (10–40 μl). The course of PCR was not influenced by the presence of naked (non-magnetic) carriers (Fig. 3). The decrease of PCR sensitivity was detected after adding naked magnetic microcarriers to the PCR mixture. It was manifested not only by the decrease of detection sensitivity but also by the appearance of non-specific ''smear'' amplicons. Testing of the effect of the particular components on the PCR course is in progress.

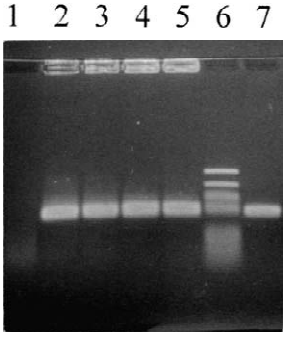

Fig. 3. Agarose gel electrophoresis of PCR products of *S. enterica* Typhimurium LB 5000 cells. The amplification was carried out in the presence of naked (non-magnetic) poly(HEMA-*co*-EDMA) carrier. Conditions: 0.8% agarose gel, TBE (45 m*M* Tris–borate, 1 m*M* EDTA, pH 8.0) buffer. Lanes: 1=control without DNA; 2–5=10–40 μl of particles; 6=DNA standards (155–970 bp); 7=positive control with purified *Salmonella* DNA. The concentration of DNA was 100 ng/μl.

3.2. Elimination of intracellular inhibitors using proteinase K immobilized on poly(HEMA-co-EDMA) microspheres

In a previous study [12], PCR was tested for the confirmation of *Salmonella* cells with untypical biochemical reactions of *Salmonella* cells or their unusual growth on differentiation media. PCR sensitivity of some *Salmonella* strains decreased if stored cell lysates were amplified or PCR products were stored before agarose gel electrophoresis. This shortcoming can not be solved by using IMS, because it is caused by the presence of intracellular inhibitors in *Salmonella* cells. According to Gibson and McKee [23], post-PCR degradation can be generated from nonpurified *Salmonella* DNA by thermostable nuclease.

A very large amount of PCR inhibitors with pronounced activity was found in the *Salmonella arizonae* 18/78 strain [9,12]. It was possible to eliminate the influence of PCR inhibitors on the course of PCR by boiling the cells for 30 min, diluting the DNA matrix, or treating the matrix with free or immobilized proteinase K on magnetic particles (1 h at 55 °C). The inhibitors of this strain were only partially eliminated by boiling for 20 and 30 min or by using free proteinase K. However, the influence of the inhibitor was fully eliminated by immobilized proteinase K. The difference in the effect of free and immobilized proteinase K can be explained as follows. If the free proteinase K is presented in the reaction mixture it can interact in the next PCR with polymerase. Its activity must be consequently suppressed after the degradation of the inhibitors. Proteinase K is activated by Ca^{2+} ions and the inhibition of proteinase K activity is not complete after addition of EDTA. The enzyme activity achieved 75% of its original value [24] as the Ca^{2+}–EDTA complex continued to interact with the enzyme which was not removed from the solution. Therefore, the authors [25] recommend use of phenylmethylsulfonyl fluoride for proteinase K inactivation. However, this compound inhibits other proteins (mainly proteases) and in addition is not appreciably soluble and stable in aqueous solutions. It is necessary to separate this compound from the sample before its use in PCR. Use of immobilized proteinase K represents thus an experimentally more susceptible procedure. The immobilized enzyme can be easily removed from the reaction mixture by a

powerful magnet, and the laborious removal of free enzyme or enzyme inhibitor is avoided.

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