

Interpolyelectrolyte Complexes of Maleic Acid Copolymers and Chitosan for Stabilization and Functionalization of Magnetite Nano- and Microparticles

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ABSTRACT: A facile method of preparation of stabilized and functionalized nano- and microparticles of magnetite by successive application of oppositely charged polymers with a regular structure of macromolecular chains (chitosan and maleic acid copolymers) onto Fe_3O_4 core are developed. This approach makes it possible to create two types of magnetite interpolyelectrolyte shells, containing carboxylic or amino groups in outer layer of shell. Composition and magnetic properties of composite particles depend on nature of the copolymer of maleic acid, reaction conditions and size of obtained particles. The carboxylic groups of copolymer in outer layer of interpolyelectrolyte shell were converted into reactive anhydride groups by heating. Thermal treatment also leads to covalent crosslinking of shell and improves stability of composites both in acidic and alkaline media. Horseradish peroxidase was successfully immobilized onto covalently crosslinked and activated microparticles of Fe_3O_4 in aqueous medium without of condensing agents. The proposed reproducible and low-cost technique does not use toxic reagents or solvents at all stages, including preparation of Fe_3O_4 , formation, activation and crosslinking, of magnetite shell, modification of activated surface of composite particles. © 2013 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2014**, *131*, 39663.

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INTRODUCTION

Magnetic nano- (MNPs) and microparticles (MMPs) especially based on magnetite are widely used in scientific researches, various fields of technology, medicine and biotechnology because of their size effects, availability of precursors, biocompatibility.^{1–12} Moreover, magnetic nanomaterials display unusual properties, such as high values of the exchange interaction and abnormally large magnetocaloric effect.¹³ Multifunctional properties of these particles allow to use them for various purposes: construction of printing devices in the form of magnetic fluids (ferrofluids),¹⁴ for magnetic storage of information,¹⁵ optoelectronic,¹⁶ spintronic,¹⁷ catalysis,¹⁸ removal of hazardous contaminants from aqueous media¹⁹ (e.g., copper ions²⁰ and radionuclides²¹), and for medical and biotechnological applications. The most common use of MNPs and MMPs in medicine includes the preparation of magnetic materials for contrast images in magnetic resonance imaging (MRI),^{8,22} systems of directional transportation and delivering of biologically active compounds and drugs,^{1,4,6,10} construction of three-dimensional “in vivo like” biotissues,²³ etc. Biotechnological applications mainly include biosensors preparation,^{8,11,24} immobilization or isolation of

drugs, proteins,^{9,25–27} nucleic acids,²⁸ cells^{8,29} (including stem cells³⁰), etc.

Although sources of magnetite MNPs and MMPs are very diverse (e.g., the biogenic magnetite of *Magnetotactic spirillum* is described³¹), usually physical, physicochemical and chemical methods^{5,7,9,12} of MNPs and MMPs preparation have been used mainly. Among them most used ones are thermolysis of precursors and synthesis in solution (microemulsionic method or coprecipitation of Fe (+2) and Fe (+3) salts in an alkaline medium). After the preparation of magnetite nano- and microparticles their surface requires protection from spontaneous aggregation and corrosion. Polymer coatings allow the modulation of properties of shell—hydrophobization or hydrophilization, functionalization of the surface of particles for desired applications. The developing strategy for construction of stable magnetite shells is the preparation of interpolyelectrolyte complex (IPEC) covering. IPECs, in particular of chitosan (the artificial polyaminosaccharide derived from chitin, available from abundant renewable resources) and anionic polyelectrolytes, are widely used in biotechnology, medicine, pharmacy, food industry, etc.³² IPECs of chitosan with polymer of monocarboxylic

(acrylic) acid as a stabilizing coatings of magnetite have been described.^{33,34} But IPECs of chitosan with alternant copolymers of dicarboxylic (maleic) acid have not been tested for stabilization and functionalization of MNPs and MMPs. The main advantages of maleic acid copolymers are: commercial availability of polymers in form of maleic anhydride copolymers or, at least, possibility of copolymer synthesis according to known procedures; easy to converse maleic anhydride groups to maleic acid ones in aqueous media. Also reverse forming of anhydride groups is possible from maleic acid residues by heating of dried copolymer. Active anhydride groups are capable of reacting with amino and hydroxyl groups of different compounds and participating in a further crosslinking of interpolyelectrolyte shell.

The aim of this study was to develop an efficient technology for the preparation of functionalized, crosslinked and activated magnetite MNPs and MMPs, applicable for biotechnological usages, by means of formation and transformation of interpolyelectrolyte shell of magnetite. To realize the aim the successive application of oppositely charged polymers (chitosan and maleic acid copolymers) onto magnetite surface was used. Two types of

coatings were proposed: copolymer of maleic acid was exposed on the outer surface of coating (Figure 1, *Type 1*) and chitosan was exposed on the outer surface of shell (Figure 1, *Type 2*). It was shown that the reinforcement and activation of magnetite shell was achieved by thermal treatment. Composition, pH-stability and magnetic properties of MNPs and MMPs were investigated as well. Also, derivatization protocol was elaborated and possible biotechnological application of MMPs was discussed.

Functionalized nano- and microparticles of magnetite were characterized by FTIR, TEM, optical microscopy, AFM, DLS, UV-VIS spectrometry, and magnetometry.

EXPERIMENTAL

Materials and Characterization Methods

Poly(ethylene-*alt*-maleic anhydride) *M* 25,000 was purchased from Monsanto (USA). Poly (*N*-vinyl-2-pyrrolidone-*alt*-maleic anhydride), *M* 40,000, was prepared following the described procedure.³⁵ The copolymers were hydrolyzed to corresponding copolymers of maleic acid by dissolving in water followed by

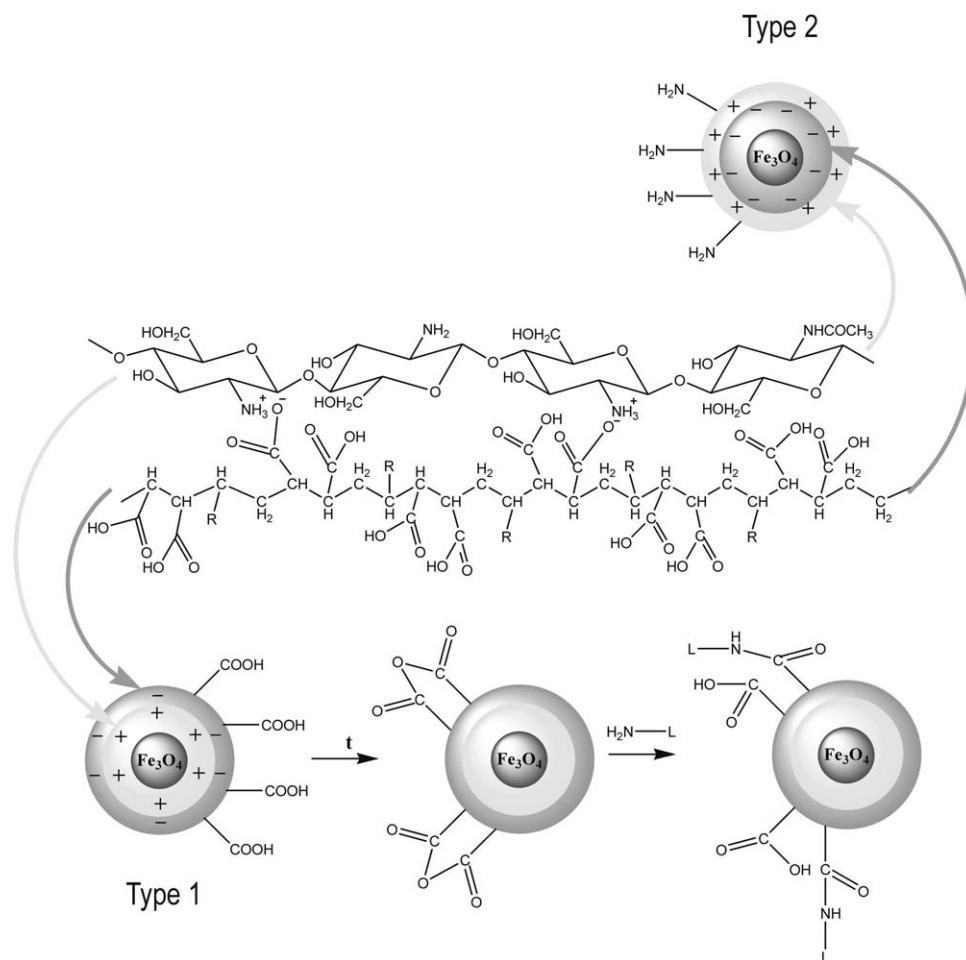


Figure 1. Schematic representation of the method used for magnetite coating, functionalization, and modification. Two types of interpolyelectrolyte coatings of magnetite were obtained by successive in two steps application of oppositely charged polymers: the shell of the lower composite (*Type 1*) includes an inner layer of chitosan and outer layer of maleic acid copolymer; an upper composite (*Type 2*) includes the maleic acid copolymer as an inner shell layer and chitosan as an outer one. The interpolyelectrolyte shell of *Type 1* composite was crosslinked thermally and maleic acid residues of copolymer were converted to maleic anhydride ones, then the amine-containing ligand was introduced into the composite (reactions below).

lyophilization. Chitosan (deacetylation degree 85%, M 90,000) was purchased from “Bioprogress” (Moscow, Russia). N^Z -Fluorenylmethyloxycarbonyl-L-Lys hydrochloride and sulfordamin B sodium salt were purchased from Fluka. Methylene blue and Eosin B were the products of Aldrich. Horseradish peroxidase (HRP) was purchased from Sigma (250 U/mg).

Composition of the magnetite composites was determined by elemental Fe, C, H, N analysis (the Laboratory for microanalysis of INEOS RAS). FTIR spectra of MNPs and MMPs were recorded with a Nicolet Magna IR-720 Fourier transform IR spectrometer (USA). Vibrating sample magnetometer (BHV-55) was used to characterize the magnetic saturations of MNPs and MMPs. UV–VIS absorption spectra were obtained with a UVIKON-922 (BRD) spectrophotometer. Transmission electron microscopy (TEM) micrographs were performed with a LEO 912 AB (Omega, Karl Zeiss; BRD) microscope operating at the accelerating voltage of 100 kV. For TEM observations, a drop of particles colloidal dispersion in water was placed onto Formvar coated copper grid and then dried. Particle size distributions were obtained by measuring of 100–300 individual particles. AFM topographic images of MNPs were obtained in tapping mode with a FemtoScan atomic force microscope (Advanced Technologies Center, Russia) with the use of commercially available Si cantilevers (NSG 11 S) with a spring constant of 11.5 N/m. The images were taken at typical scan rate of 0.5–1.0 Hz and processed with FemtoScan online software. DLS experiments were conducted on an ALV/DLS/SLS-5022F spectrometer (ALV-GmbH, Langen, Germany) equipped with an ALV5000 digital time correlator and 22 mW UNIPHASE He-Ne laser ($\lambda_0 = 632$ nm). Measurements were made at 25°C. Optical microscopy was performed using an Eclipse H550S (Nikon, Japan) microscope, equipped with a Kodak DC 120 Digital Camera. pH-stability of magnetite polymer coatings was studied using a spectrofluorimeter “Fluorat®-02-panorama” (Lumex, S.-Peterburg, Russia).

Synthesis of Fe_3O_4 Nanoparticles

Magnetite (Fe_3O_4) was prepared by dehydration and coprecipitation of freshly prepared Fe^{+2} and Fe^{+3} hydroxides formed from their salts (taken in 1:2 molar ratio) in an alkaline solution following the described method.³⁶ Briefly: 1.4 g $\text{Fe}_2(\text{SO}_4)_3 \cdot 9\text{H}_2\text{O}$ and 0.7 g $\text{FeSO}_4 \cdot 12\text{H}_2\text{O}$ were dissolved in 25 mL of deionized (DI) water, then 10 mL 25% aqueous ammonium solution in 40 mL of water was added under sonication (22 kHz) of reaction mixture. Then the reaction mixture was sonicated for 2 min. The black precipitate of magnetite was collected with the use of a permanent magnet (the magnetic field strength 2000G), washed using DI water several times until pH neutrality of the effluent and was used immediately after the preparation. Obtained dispersion (1.80 g) contained ~0.19% of dry Fe_3O_4 .

Preparation of Magnetite/Polymer Composites

Preparation of magnetite/chitosan composite ($\text{Fe}_3\text{O}_4/\text{CS}$): freshly prepared magnetite (0.50 g dispersion in water, containing 0.11 g of dry Fe_3O_4) was mixed with 50 mL DI water, sonicated (22 kHz) for 2 min and then 5 mL of 2% chitosan solution in CH_3COOH (1%) was added to the mixture. The reaction

mixture was additionally sonicated for 2 min. Magnetic composite was collected with the use of the permanent magnet and washed using DI water. Nanoparticles of $\text{Fe}_3\text{O}_4/\text{CS}$ composite (MNPs) were separated from the microparticles (MMPs) by centrifugation at 3000 rpm for 5 min and then MNPs and MMPs were lyophilized. The yield of MNP fraction was 6%, the yield of MMPs—94%.

Magnetite/poly(ethylene-*alt*-maleic acid) ($\text{Fe}_3\text{O}_4/\text{EM}$) and magnetite/poly(*N*-vinyl-2-pyrrolidone-*alt*-maleic acid) ($\text{Fe}_3\text{O}_4/\text{VM}$) composites were prepared following the above described protocol with the use of 5 mL 2% copolymer solution in water (pH 5).

Preparation of magnetite/chitosan/copolymer composites ($\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$): $\text{Fe}_3\text{O}_4/\text{CS}$ MNPs or MMPs (0.10 g), were dispersed in 50 mL of DI water, sonicated (22 kHz) for 2 min and then 1 mL 2% aqueous solution of EM (pH 5) was added to the mixture. The reaction mixture was sonicated for 2 min. The composites were collected with the use of the permanent magnet, washed using DI water and lyophilized. Yield of MMPs was 0.11 g, MNPs—0.14 g.

MNPs or MMPs of $\text{Fe}_3\text{O}_4/\text{EM}/\text{CS}$ or $\text{Fe}_3\text{O}_4/\text{VM}/\text{CS}$ were prepared following the above described protocol with the use of $\text{Fe}_3\text{O}_4/\text{EM}$ or $\text{Fe}_3\text{O}_4/\text{VM}$ and 1 mL 2% chitosan solution in CH_3COOH (1%). Yield of MMPs fractions was 0.12 and 0.13 g, respectively.

IPEC CS/EM as the model compound for FTIR spectra was prepared by the mixing of the aqueous solutions of equimolar amounts of polycation (CS) and polyanion (EM) at pH 5. The precipitate was washed with water and lyophilized.

Evaluation of Content of Outer Polymer Layer in Interpolyelectrolyte Shells by Dye Displacement Method

Content of polymer in outer layer of interpolyelectrolyte shell of nano- and microcomposite particles $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ was assessed by dye displacement method. The dye—methylene blue (MB)—from complex VM–MB was displaced by chitosan of $\text{Fe}_3\text{O}_4/\text{CS}$ composite. Complex VM–MB was obtained as follows: aqueous solution of copolymer (0.2 mL, 0.7×10^{-3} mol/L) was added to aqueous solution of MB (1.8 mL, 2.0×10^{-5} mol/L). Optical density at $\lambda = 665$ nm was measured and was admitted as the initial optical density (D_0). VM–MB complex was mixed with precisely weighted quantities of $\text{Fe}_3\text{O}_4/\text{CS}$, taken in increasing amounts, and the optical density D_i of the solution containing MB (released from VM–MB) was measured. Following the above described protocol the content of outer chitosan layer in $\text{Fe}_3\text{O}_4/\text{MAC}/\text{CS}$ was evaluated by eosin (E) displacement from CS–E complex by precisely weighted quantities of $\text{Fe}_3\text{O}_4/\text{VM}$ or $\text{Fe}_3\text{O}_4/\text{EM}$, respectively, in aqueous solution at $\lambda = 515$ nm.

For evaluation of the amount of functional groups which are not involved into interpolyelectrolyte interaction the titration of MMPs by the anionic (eosin) or cationic (methylene blue) dyes was carried out. Under titration the increasing volumes of solution of dye were added to precisely weighted amounts of magnetite composite until the process of discoloring of solution had stopped.

Stability Tests of Composites

Stability of MMPs containing labeled polymers in the shell was determined by a fluorimetric method. The parent polymers

were labeled and then labeled polymers were applied for magnetite covering.

Preparation of N^{α} -Fluorenylmethoxycarbonyl(FMOC)-L-Lysine-labeled poly(ethylene-*alt*-maleic acid) (FMOC-EM): EM solution (1.72 g, 13.7 mmol) in 15 mL of dry dimethylformamide (DMF) was mixed with the solution of N^{α} -FMOC-L-lysine (0.095 g, 0.137 mmol) in 2 mL of DMF. The resultant solution was stirred for 5 h at room temperature. Then, the solution was diluted with DI water, dialyzed against DI water (5L \times 3), and lyophilized. The degree of substitution was calculated from the absorbance of FMOC-EM solution in DI water at $\lambda = 300$ nm and found to be 1 mol %.

Chitosan was labeled by Sulforodamin B (SR) following the described protocol³⁷ (CS-SR), and the degree of labeling for CS-SR was found to be 0.005 mol %. Magnetite composite containing labeled polymer (10 mg) was placed in 3 mL buffer (0.05M glycine-HCl pH 2 or 0.05M glycine-NaOH pH 9) and the mixture was kept for period 1–500 h at 30°C. The MMPs were periodically separated with the use of the permanent magnet, and fluorescent response of the supernatant was measured at $\lambda_{em} = 586$ nm for CS-SR ($\lambda_{ex} = 568$ nm) or at $\lambda_{em} = 260$ nm for FMOC-EM ($\lambda_{ex} = 306$ nm). We analyzed the changes in the fluorescence spectra occurring in the supernatant solution after separation of magnetic-field component from the reaction mixture with the use of the permanent magnet, and then calculated the degree of destruction of MMPs shells. As the control labeled polymers containing MMPs were stored in distilled water (pH 7) at room temperature for period 6 months.

Thermal Activation and Crosslinking of Magnetite Shells, HRP Immobilization onto Activated Composite Particles

For activation and crosslinking of magnetite interpolyelectrolyte shell, a powdery sample of MMPs $Fe_3O_4/CS/EM$ (0.030 g) was heated at 110°C for 3 h over P_2O_5 (under vacuum) and then cooled to room temperature. For FTIR spectrum the model compound (IPEC CS/EM) was heated under the same conditions.

The activated MMPs were added to 1 mL solution of HRP (2 mg/mL) in Milli-Q quality grade water. The mixture was vigorously stirred for 5 h, and the magnetic particles were collected and washed successively with the water until the absence of peroxidase activity in the effluent, using the permanent magnet for MMPs collection. The contents of immobilized HRP onto activated MMPs was calculated, and peroxidase activity of native and immobilized HRP was determined at $\lambda = 420$ nm by the observing of the oxidation of ferrocyanide $K_4Fe(CN)_6$ to ferricyanide $K_3Fe(CN)_6$ by hydrogen peroxide following the described method.³⁸

RESULTS AND DISCUSSION

Magnetite nanoparticles obtained by the well-known method of coprecipitation of Fe (+2) and Fe (+3) salts in an alkaline medium³⁶ were covered by polymers immediately after the preparation. In this research the surface stabilization and functionalization of magnetite (Fe_3O_4) particles were achieved by the formation of interpolyelectrolyte shell on Fe_3O_4 core.

Surface activation and stronger fixation of the shell were achieved by thermal treatment of composite particles (Figure 1).

Two types of reversed interpolyelectrolyte shells were obtained by the reversed order of application of oppositely charged polymers to magnetite particles. Each type of interpolyelectrolyte coating was formed in two stages: a) formation of inner polymer layer (“monopolymer” covering) and b) creation of interpolyelectrolyte shell.

Formation of Magnetite/Polymer Composites

Nanoparticles of magnetite stabilized with one of hydrophilic polymers (chitosan or poly(acrylic acid)) are well-known. Magnetite nanoclusters stabilized with poly(acrylic acid) have been synthesized in organic phase at 220°C in a nitrogen atmosphere.³⁹ It has been shown that a part of carboxylic groups of poly(acrylic acid) are strongly coordinated with cations of the magnetite surface, while uncoordinated carboxylic groups extended into the aqueous solution.³⁹ Chelation of the amino groups of chitosan with polar Fe_3O_4 nanoparticles was confirmed as well, but the binding of magnetite with chitosan was not strong enough in protonated chitosan solution, so chitosan hydrogel, instead of chitosan solution, has been used in the synthesis of Fe_3O_4 composite so far.⁴⁰

At first stage of our work the freshly prepared Fe_3O_4 particles were covered by one of the polymer, CS or MAC. The mild conditions for the formation of magnetite monopolymer shells were used: aqueous media, room temperature and ultrasonic treatment of reaction mixture. By preliminary experiments it was found that the pH optimum for polymer layer formation was 3.5 for chitosan and for MAC, 5.5, and optimal concentration of solutions of polymer was 1–2%. As to poly(ethylene-*alt*-maleic acid), the concentration used did not exceed its critical concentration of aggregation (below 2% wt.).⁴¹ It was noted that more aggregated particles were formed at higher polymer concentrations and lower mixing rates. MNPs and MMPs fractions were separated by centrifugation. Table I shows the composites of magnetite covered by one of two polymers only and their characteristics (samples 1–6). The contents of organic (polymer) phase in the composites magnetite/chitosan (Fe_3O_4/CS), magnetite/poly(ethylene-*alt*-maleic acid) (Fe_3O_4/EM), magnetite/poly(*N*-vinyl-2-pyrrolidone-*alt*-maleic acid) (Fe_3O_4/VM) were in the range of 24–43% wt. and were mainly depended on sizes of particles but not on nature of polymer. MNPs contained maximal amounts of polymer (39–43% wt.) in shells since cores of MNPs were smaller in sizes and greater in the surface area than those of MMPs.

It should be noted that *in situ* technique used for the preparation of magnetite/CS (synthesis of Fe_3O_4 particles in the presence of polymer) resulted in higher heterogeneity in particle size distribution of the composites and lower magnetic saturation values in comparison with the method described above. Moreover *in situ* technique of stabilization of magnetite by MAC resulted in the precipitation of ferric-MAC salts. As a result we refused *in situ* technique in our work.

In contrast to monopolymer covering of Fe_3O_4 the bilayer one could be diminish the susceptibility of composites to the

Table I. Composition and Properties of Magnetite Composites

Sample	Particle type	Particle mean size (standard deviation)	Polymer content: Inner layer/outer layer (wt %) ^a	Saturation magnetization (gauss cm ³ /g)
1	Fe ₃ O ₄ /CS	25(13) ^b /48(7) ^c nm	39.0/-	12.5
2	Fe ₃ O ₄ /CS ^d	15(10) ^e μm	29.2/-	48.3
3	Fe ₃ O ₄ /VM	38(18) ^b /63(8) ^c nm	42.1/-	12.1
4	Fe ₃ O ₄ /VM	22(10) ^e μm	24.0/-	39.0
5	Fe ₃ O ₄ /EM	25(15) ^b /54(9) ^c nm	43.2/-	12.0
6	Fe ₃ O ₄ /EM ^d	16(13) ^e μm	29.5/-	43.0
7	Fe ₃ O ₄ /CS/VM	34(14) ^b /75(10) ^c nm	38.0/33.0	6.5
8	Fe ₃ O ₄ /CS/VM ^f	28(11) ^e μm	19.3/1.2	49.2
9	Fe ₃ O ₄ /CS/EM ^d	26(12) ^e μm	18.0/2.7	-
10	Fe ₃ O ₄ /CS/EM	25(15) ^e μm	23.7/2.1	41.0
11	Fe ₃ O ₄ /CS/EM	88(10) ^c nm	39.0/28.0	-
12	Fe ₃ O ₄ /CS/EM ^d	22(14) ^e μm	20.4/1.3	-
13	Fe ₃ O ₄ /VM/CS	20(11) ^b /70(9) ^c nm	41.7/29.2	-
14	Fe ₃ O ₄ /EM/CS	23(12) ^b nm	40.0/30.0	-

^aThe composition was determined by elemental Fe, C, H, N analysis.

^bAFM method.

^cDLS method.

^dFluorescently labeled sample.

^eOptical microscopy method (the samples also contained the particles <5 μm in diameter).

^fCrosslinked composite shell.

external factors (pH, ionic strength, etc.). The creation of interpolyelectrolyte complex shells is one of the ways of stabilization of different nano- and microparticles. There are several procedures for the preparation of IPEC: polymerization of ionic monomers on oppositely charged polymeric matrices or mixing of aqueous solutions of polycations and polyanions.

Earlier, for stabilization of magnetite an interpolyelectrolyte complex of CS with monocarboxylic acid polymer [polyacrylic acid (PAA)] was used.^{33,34} Magnetic Fe₃O₄/CS/PAA microspheres were prepared by graft-polymerization of acrylic acid onto Fe₃O₄/CS particles. CS–PAA complex proved to be a good stabilizer of the well-dispersed suspension of paramagnetic Fe₃O₄.

In comparison with the described method,³³ in this work, the outer (second) layer of interpolyelectrolyte magnetite shell was formed in water solution by means of adsorption of the oppositely charged polymer onto magnetite/monopolymer composite particles. Particles of *Type 1* contain MAC in the outer layer of interpolyelectrolyte shell, while particles of *Type 2* contain MAC in the inner one (Figure 1, Table I, samples 7–14). The concentrations of polymers used in the construction of both inner and outer layers of interpolyelectrolyte shell were identical, and pH values used for the formation of outer layer were in the range of 4–5. As it was previously shown, pH-optimum for formation of polyelectrolyte complex CS–MAC was about 4–5 since the degrees of ionization of both interacting amino and carboxylic groups under these conditions are high. At pH = 5 the degree of protonation of amino groups of chitosan is 98%, and the degrees of ionization of first and second carboxylic groups of

EM are 97% and 5%, respectively. The same values for VM are 95% and 2%, respectively.⁴² Also it has been found that the process of CS and MAC interaction is cooperative, and the effective binding constants for reactions of CS with poly(*N*-vinyl-2-pyrrolidone-*alt*-maleic acid), poly(styrene-*alt*-maleic acid) or poly(ethylene-*alt*-maleic acid) are in the range of (1–3) × 10⁻⁴ L/mol.⁴³

Prepared composites contained 25–75% of magnetite and exhibited the saturation magnetization values of 6–50 gauss × cm³/g. The latter values decreased with the increasing of the content of organic phase in the composites (Table I). Magnetization values were low for MNPs covered by one of polymers (samples 1, 3, 5) only and they were minimal for MNPs stabilized with interpolyelectrolyte shells (sample 7). The saturation magnetization values of MNPs containing large magnetic core were maximal (samples 2, 4, 6, 8, 10).

Thermal Activation and Crosslinking of Magnetite Shells, HRP Immobilization on Activated Composite Particles

For stronger fixation of interpolyelectrolyte coating of composite particles their thermal treatment was carried out. The heating causes the transformation of ionic bonds between amino groups of CS and carboxylic groups of MAC to covalent amide (imide) bonds. Under these conditions dicarboxylic acid residues of outer copolymer layer untouched during the interpolyelectrolyte interaction were converted to anhydride groups⁴⁴ [Figure 1; FTIR spectra (Figure 2, 3)]. Another method for preparation of crosslinked, anhydride containing shell was the formation of outer layer on Fe₃O₄/CS composite particles in the waterless organic solvent (DMF) with the use of MAC taken in

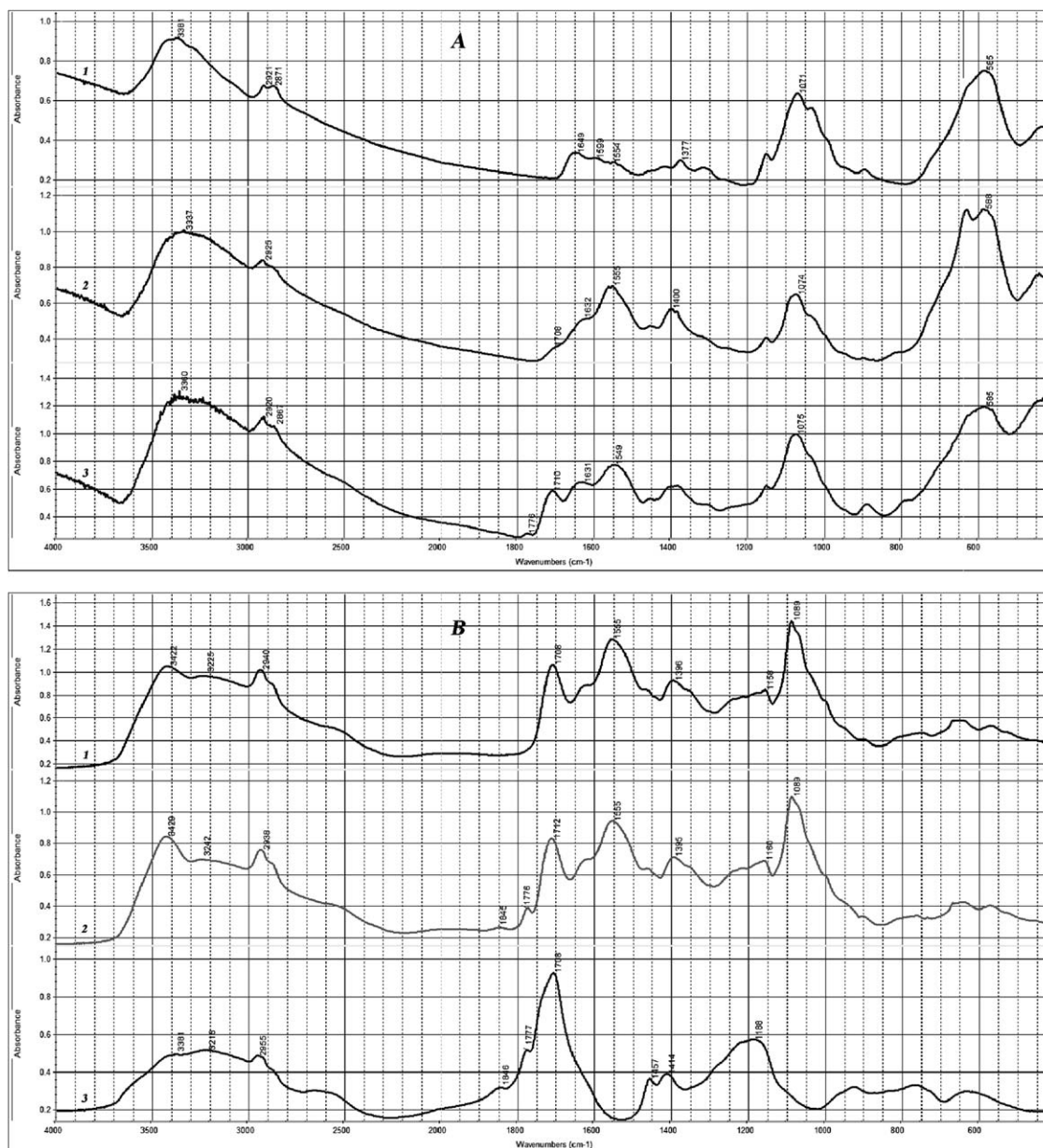


Figure 2. A: FTIR spectra of magnetite composites: $\text{Fe}_3\text{O}_4/\text{CS}$ (1), $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ (2), $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ after thermal treatment (3). B: FTIR spectra of model compounds: IPEC CS/EM (1), IPEC CS/EM after thermal treatment (2), EM (in the form of copolymer of maleic anhydride) (3).

the form of maleic anhydride copolymer. The proposed method is a simple alternative approach for increasing of stability of shells by means of their covalent crosslinking with bifunctional agents or by using of condensing agents.^{34,45}

The possibility of a practical application of the composites for biotechnological purposes was demonstrated for MMPs containing the interpolyelectrolyte shell. We preferred MMPs instead of MNPs because of more convenient working conditions in the case of using of MMPs – faster sedimentation of MMPs in the magnetic field and easier separation of liquid phase. Functional groups exposed on the outer surface of MMPs— $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ (Table I, 8) and $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ (Table I, 11), were activated by heating before enzyme immobilization. The reactivity of resulting anhydride groups of MMPs allowed the binding of horseradish

peroxidase (HRP) via amide bonds formation under mild conditions in aqueous medium without the usage of any condensing agents. The contents of HRP immobilized onto $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ and $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ were found to be 10 mg/g (0.25 $\mu\text{mol/g}$) and 1.6 mg/g, correspondingly; the relative specific HRP activity was 80% and 75%, respectively. Earlier described magnetized silica-based microparticles (5 μm) contained immobilized HRP in amount of 0.1–0.3 $\mu\text{mol/g}$.⁴⁶

Characterization of Magnetite Composites

Figure 2(A) shows FTIR spectra of magnetite covered by one of the polymers, IPEC shell and crosslinked shell. The resolutions of FTIR spectra as regards to characteristic absorption bands of polymers in composites are not very clear since composite particles contain large amounts of Fe_3O_4 . To clarify FTIR spectra of

organic phase of composites the model samples, containing only the polymers of shells, were prepared and analyzed. Figure 2(B) shows FTIR spectra of the model compounds—stoichiometrical IPEC, heated IPEC and copolymer of maleic anhydride.

The peaks at $\nu = 585\text{--}588\text{ cm}^{-1}$ in all spectra [Figure 2(A)] were assigned to characteristic band of Fe–O within Fe_3O_4 .⁴⁷ The spectrum of $\text{Fe}_3\text{O}_4/\text{CS}$ [Figure 2(A), 1] contains the characteristic absorption bands at $3400\text{--}3200\text{ cm}^{-1}$ which were assigned to the stretching vibrations of OH and NH. CH stretch vibrations were at $2921, 2871\text{ cm}^{-1}$, C–O–C—at 1071 cm^{-1} , 1649 cm^{-1} (amide I), $1554, 1599\text{ cm}^{-1}$ (amide II), and 1377 cm^{-1} (CH_3) (CH_3CONH -groups of chitosan). IR spectrum of CS-coated magnetite particles was described earlier.⁴⁸ In the spectrum of $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ [Figure 2(A), 2] and IPEC CS/EM [Figure 2(B), 1] the absorption bands in the region of $1632\text{--}1708\text{ cm}^{-1}$, $\nu_{\text{COO}^-} = 1555, 1400\text{ cm}^{-1}$, and $\nu_{\text{C=O}} = 1074\text{ cm}^{-1}$ belonging to CS–MAC interpolyelectrolyte complex were presented earlier.⁴⁴ The thermal treatment of $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ [Figure 2(A), 3] and CS/EM [Figure 2(B), 2] led to crosslinking of shell CS/EM. As a result the absorption band of amide groups at 1549 cm^{-1} , the adsorption band at $\nu_{\text{C=O}} = 1710\text{ cm}^{-1}$ and 1776 cm^{-1} appeared. The last two bands indicate the appearance of the cyclic structure of succinic anhydride and partially succinimide groups similar to those which appeared after the thermal treatment of CS–MAC interpolyelectrolyte complex.⁴⁴ The cyclic structure of succinic anhydride was

sharply defined also in the spectrum of poly (ethylene-*alt*-maleic anhydride) [Figure 2(B), 3].

Transmission electron microscopy (TEM) shows the presence of magnetite nanocrystals and nanoparticles in the form of flocculated clusters in cores of composite particles after all steps of formation of polymer shells. Histograms of size distributions of Fe_3O_4 nanocrystals (individual ones and in clusters) in the mono- and bilayer covered magnetite particles are represented in TEM images (Figure 3, a1, b, c). Nanocrystals of magnetite in all magnetite-polymer composites are roughly spherical and their sizes range in $3\text{ nm} < D_{\text{TEM}} < 15\text{ nm}$. The formation of iron oxide clusters of individual nanocrystals is governed by a balance of van der Waals and electrostatic interactions. Moreover, polymers are able the clustering of the primary magnetite nanocrystals upon their interaction with polymer chains. Also, it is necessary to take into consideration the opportunity of alteration of cluster sizes during the preparation of samples for TEM, since their morphological appearance can be perturbed from that in the aqueous dispersion. The sizes of discovered clusters in cores of the samples varied from 20 to 400 nm (Figure 3, a2) independently of stage of formation of polymer shell.

The cores of MNPs apparently contained individual nanocrystals and small nanoclusters while MMPs ones contained the clusters of Fe_3O_4 mainly. The differences in ratio of the amounts of nanocrystals and clusters were in accordance with these [about 1: (8–9)] of amounts of MNPs and MMPs

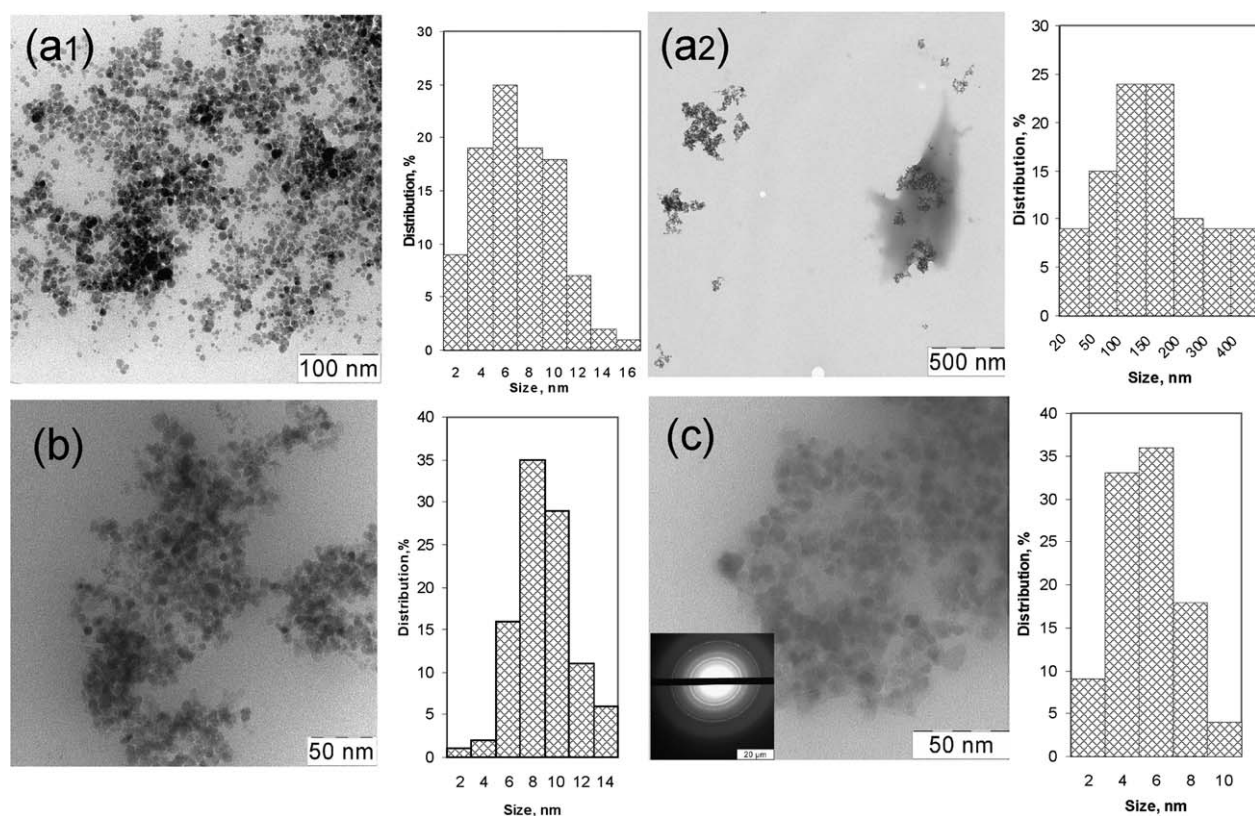


Figure 3. TEM micrographs of magnetite composites: (a1, a2) $\text{Fe}_3\text{O}_4/\text{CS}$ (Table I, 1), (b) $\text{Fe}_3\text{O}_4/\text{VM}$ (Table I, 3), (c) $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ (Table I, 7), inset shows the electron diffraction pattern. Size distributions: nanocrystals (a1, b, c) and clusters (a2).

obtained at the experimental conditions used (polymer concentrations, pH of medium, velocity and time of mixing).

The crystalline nature of nanoparticles of Fe_3O_4 , for example, for composite particles $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ is illustrated by the electron diffraction (ED) image (Figure 3, *c*, inset). The strong lattice rings in ED image can be indexed to (220), (311), (400), (422), (511), and (440) planes of the standard magnetite system (JCPDS card No: 19-0629).

As it was expected, average size (diameter) of magnetite composite particles reasonably exceeded that of metal oxide particles themselves. Thus, the average size of MNPs (optical microscopy data, see Figure 4) in the case of magnetite with monopolymer shell was 15–20 μm (standard deviation of 10 μm) (Table I, 2, 4, 6), and that of $\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ and $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ 22–28 μm (standard deviation of 11–14 μm) (Table I, 8–11). As it is seen after application of second polymer layer on magnetite/monopolymer microparticles small increasing in size of particles was occurred only.

The sizes of MNPs determined by DLS are represented in Table I (samples 1, 3, 5, 12). Sizes of double-layered MNPs also slightly exceed the sizes of mono-coated nanoparticles. The data of DLS sizes determined for MNPs naturally exceeded the size of these particles determined by AFM method.

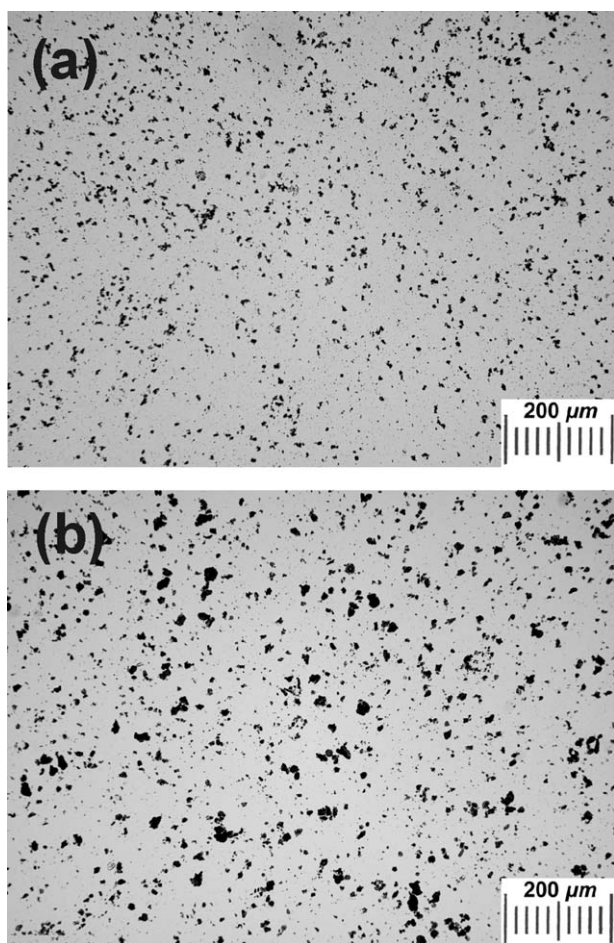


Figure 4. Optical microscopy micrographs of magnetite nanocomposites: (a) $\text{Fe}_3\text{O}_4/\text{CS}$ (Table I, 2) and (b) $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ (Table I, 10).

Figure 5 shows AFM images of magnetite composites containing both monopolymer and interpolyelectrolyte shells. It is seen from the images that the shapes of most of the particles were almost spherical and the average diameters of particles were in the range of 5–100 nm. The whole array of particles is divided into chosen size ranges (Table II). Table II shows that the sizes of magnetite particles stabilized by one of polymer alter slightly after the treatment of the particles with an oppositely charged polymer, as it was shown by DLS studies and optical microscopy.

Apparently the second layer was formed by the polymer molecules in their expanded conformations, and this layer did not form “braches” or “mushrooms” on the surface of particle. Here we could assume that during the formation of IPEC amino groups of chitosan glucosamine units and carboxylic groups of maleic acid residues of MAC are not quantitatively involved into interpolyelectrolyte interaction. This assumption is supported by the model of stoichiometrical IPEC complex (Figure 1), which takes into account the regularity of the structure of macromolecular chains of the interacting polymers and their steric conformation. Also this model takes into consideration the distances between NH_2 -groups of two neighboring units of glucosamine in chitosan ($\approx 5.15 \text{ \AA}$)⁴⁹ and the distances between maleic acid residues of MAC ($\approx 5.02 \text{ \AA}$). The latter value is calculated for the “ideal” polymer chain, where the distance between the chain carbon atoms is 1.54 \AA and the angle between the links 109°, assuming that the polymer chain is similar to that of polyethylene. In accordance with the model, only every second amino group of chitosan corresponds sterically and can be bound with the carboxyl group of the every second residue of maleic acid in MAC only.

Real molecular structure of polymers in the solution is sensitive to solvent, temperature, ionic strength, and pH. For example, the dichroism spectrum of chitosan-aspartic acid complex indicates that chitosan adopts the helical (left-handed) conformation.⁵⁰ Ohno described the pH-induced conformational transitions of maleic acid and α -methylstyrene copolymer from the compact form to the coil.⁵¹

For a number of magnetite/monopolymer composites the effective amount of second polymer layer which can be involved in interpolyelectrolyte shell is assessed by the dye displacement method. Amino groups of CS of composite $\text{Fe}_3\text{O}_4/\text{CS}$ displaced the methylene blue (MB) from VM–MB salt into solution, and carboxylic acid groups of VM of $\text{Fe}_3\text{O}_4/\text{VM}$ composite displace eosin (E) from CS–E salt. The maximal optical density of the dye was used as a point of saturation of $\text{Fe}_3\text{O}_4/\text{monopolymer}$ composite shell by the second polymer. And at this point, the effective amount of second polymer layer was applied onto the surface of magnetite/monopolymer composite particles. In these experiments we use diluted solutions of polymers. Surface of magnetite/monopolymer composite particles after the ousting of the dye bound the oppositely charged polymer. The results show that the amount of introduced second polymer layer increased with decreasing of particle size from micro- (Table III, samples 2, 4) to nanoparticles (Table III, samples 1, 3, 5). In some cases a part of the total amount of the first polymer layer is able to participate in the interpolyelectrolyte interaction only.

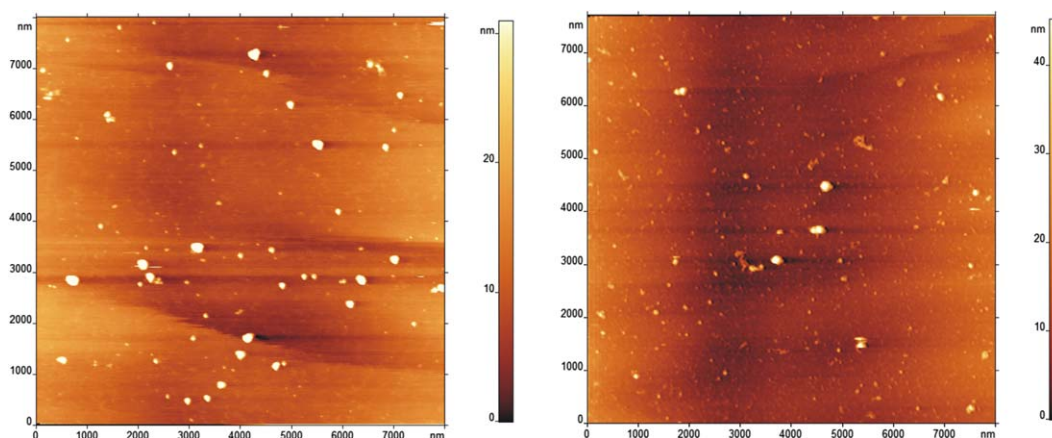


Figure 5. AFM images of magnetite nanocomposites: $\text{Fe}_3\text{O}_4/\text{EM}$ (Table I, 5)—left image; $\text{Fe}_3\text{O}_4/\text{EM}/\text{CS}$ (Table I, 14)—right image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In the case of MMPs (samples 2, 4), about 2.5–3.0% of total amount of polymer of the first layer are connected with the second polymer layer. This approach allows assessing the amount of oppositely charged polymer required for the construction of outer polymer layer taking into account the size of magnetite/monopolymer composite particles.

To evaluate the amount of functional groups which are not involved into interpolyelectrolyte interaction and can be used for modification the titration of MMPs by the anionic (eosin) or cationic (methylene blue) dyes was carried out. The titration

showed that a sufficient amount of functional groups of outer polymer layer available for connection with the dye. It should be noted that some amount of the functional groups of inner layer of magnetite interpolyelectrolyte shell also were available for titration. For example, even in the case of crosslinked MMPs $\text{Fe}_3\text{O}_4/\text{CS}/\text{EM}$ (after hydrolysis of anhydride groups) 12.4 $\mu\text{mol/g}$ of maleic acid residues and 0.8 $\mu\text{mol/g}$ amino groups were revealed. Previously it was shown that during the covering of different surfaces with the polyelectrolyte complex composed of chitosan and poly (*N*-vinyl-2-pyrrolidone-*alt*-maleic acid) by layer-by-layer method, it was shown that the

Table II. Particle Size Distribution from the AFM Data for MNPs with “Monopolymer” and Interpolyelectrolyte Coatings

Composite (sample number in Table I)	Size range (nm)	Mean radius (standard deviation), nm	Particle content (%)
$\text{Fe}_3\text{O}_4/\text{EM}$ (5)	5–20	10 (5)	61
	20–100	43 (19)	36
	100–200	120 (13)	2
$\text{Fe}_3\text{O}_4/\text{EM}/\text{CS}$ (14)	5–20	11 (5)	55
	20–100	36 (17)	43
	100–200	107 (5)	2
$\text{Fe}_3\text{O}_4/\text{VM}$ (3)	5–20	17 (2)	8
	20–100	38 (18)	72
	100–200	141 (28)	12
	>200	316 (155)	8
$\text{Fe}_3\text{O}_4/\text{VM}/\text{CS}$ (13)	5–20	9 (4)	75
	20–100	31 (11)	25
	100–200	–	–
$\text{Fe}_3\text{O}_4/\text{CS}$ (1)	5–20	12 (5)	55
	20–100	39 (18)	43
	100–200	134 (33)	2
	>200	531 (230)	1
$\text{Fe}_3\text{O}_4/\text{CS}/\text{VM}$ (7)	5–20	10 (5)	67
	20–100	39 (14)	31
	100–200	131 (31)	2

Table III. Content of Polymer in Outer Layer of Magnetite Composite Interpolyelectrolyte Shell by the Dye Displacement Method

Initial composite (sample number, Table I)	Polymer content in initial composite (mmol/g)	Polymer of outer layer	Polymer content in outer composite layer by the dye displacement method (mmol/g)
Fe ₃ O ₄ /CS (1)	2.21	VM	1.75
Fe ₃ O ₄ /CS (2)	1.65	VM	0.041
Fe ₃ O ₄ /VM (3)	2.38	CS	1.70
Fe ₃ O ₄ /VM (4)	1.05	CS	0.034
Fe ₃ O ₄ /EM (5)	2.98	CS	1.80

outer copolymer layer contained sufficient amount of carboxylic groups, capable of bearing the specific to plasminogen ligand.⁴⁴

The obtained magnetite microparticles were stable longer than 6 months at physiological values of pH, and MMPs were more stable than MNPs. The presence of negative or positive charges on nanoparticles was beneficial for their colloidal stability because of electrostatic repulsion forces. Previously it was shown earlier that maghemite NPs protectively coated with IPEC in two layer-by-layer deposition steps with poly(ethylene imine) and poly[(ethylene oxide)-block-poly(glutamic acid)] were stable in physiological salt solution for longer than 6 months.⁵²

The stability of prepared in our work MMPs covered by one of the polymer or interpolyelectrolyte shell was also evaluated under conditions, more severe than these at physiological values of pH. At first, stability of MMPs has been tested in highly acidic medium in which both parent polymers are soluble and the degradation of the IPEC are occurred.⁴² Additionally, the measurements were carried out in highly alkaline aqueous solution, where MACs are soluble while chitosan is insoluble. For these experiments, the parent polymers were preliminary labeled

and then they were applied for covering of magnetite. The stability was defined as a percentage of content of the labeled polymer in solution to its content in the initial MMPs. Histogram (Figure 6, 1) shows that a significant degradation of polymer shell of Fe₃O₄/CS took place during 100 h at pH 2 (labeled CS was monitored in the solution). In the case of composite Fe₃O₄/CS/EM (Figure 6, 2, pH 2) the degradation of interpolyelectrolyte coating was somewhat lower. As it was shown above (the composite titration experiments) both monopolymer and interpolyelectrolyte magnetite coatings contained unbound amino groups of CS, and these groups apparently contributed to the hydrolytic instability of composites. The crosslinked composite Fe₃O₄/CS/EM (Figure 6, 3, pH 2) was found to be practically stable at these conditions.

The same tendency (but significantly less pronounced) was observed during the hydrolysis of composites when the labeled copolymer was monitored in the solution (Figure 6, black columns). Low values of degree of degradation estimated by monitoring of labeled copolymer in the solution can be explained by a strong adhesion of carboxylic groups of polymer

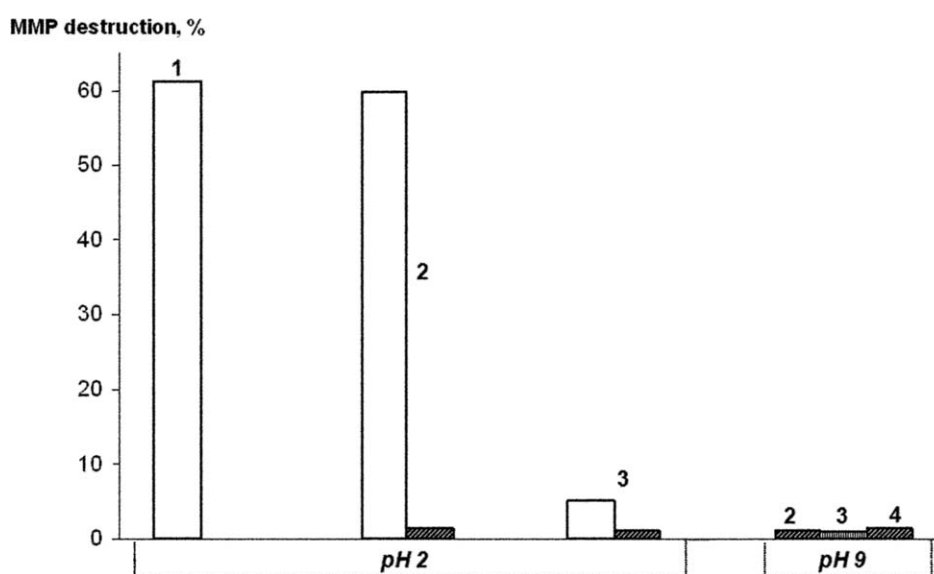


Figure 6. Magnetite microparticles shell destruction (100 h): 1—Fe₃O₄/CS (Table I, 2), 2—Fe₃O₄/CS/EM (Table I, 9), 3—Fe₃O₄/CS/EM (crosslinked) (Table I, 12), and 4—Fe₃O₄/EM (Table I, 6). Histogram bars: white columns—CS-SR detection; black columns—FMO-EM detection.

to magnetite.^{39,53} It was shown that carboxylic groups of polyacids form relatively stable complexes with atoms of iron of magnetite through bidentate bonding.⁵⁴ Besides the destruction of protective coating may cause the oxidation of magnetite as well:



and the liberated ions of iron can form insoluble complexes with residues of dicarboxylic acid of copolymer $\text{R}(\text{COOH})_2$.

In alkaline media, CS was not detected in the solution. As to copolymer, its amount in the solution did not exceed 5% for all samples during the observation time (Figure 6, columns 2, 3, 4, pH 9).

CONCLUSIONS

Here, we reported a simple procedure for preparation of stabilized and functionalized magnetite nano- and microparticles by successive application of oppositely charged polymers having regular structures of macromolecular chains (chitosan and maleic acid copolymers) onto Fe_3O_4 core. The proposed approach makes it possible to create magnetite interpolyelectrolyte shells of two reversed types containing different functional groups on outer shell layer—carboxylic or amino groups. Also the effect of different factors on the formation, structure and properties of obtained magnetite particles covered by one of the polymers and interpolyelectrolyte shells is demonstrated. The structure and composition of IPEC shells, magnetic properties of composites depended on nature of maleic acid copolymer, reaction conditions and size of core of synthesized composite particles. The prepared composites contained 25–75% magnetite and exhibited the saturation magnetization values of 6–50 gauss $\times \text{cm}^3/\text{g}$, and the latter value decreased with the increase of the content of organic phase in the composites (from micro- to nanoparticles).

The used analytical procedures allowed us to assess the amount of functional groups on the surface of composite particles. It was shown that the outer copolymer layer contained sufficient amount of functional groups available for subsequent modification. Surface-exposed maleic acid residues of copolymer were transformed to reactive anhydride groups by means of thermal activation followed by covalent crosslinking of the shell. Ion-stitched IPEC shells of magnetite microparticles were stable in aqueous media at physiological values of pH and the covalently crosslinked shells—in highly acidic and alkaline solutions.

Horseradish peroxidase was successfully immobilized onto activated and covalently crosslinked $\text{Fe}_3\text{O}_4/\text{CS}/\text{MAC}$ microparticles in aqueous medium without usage of any outer condensing agents. The proposed reproducible and low-cost technique doesn't use toxic reagents and solvents at all stages, including the preparation of Fe_3O_4 , and formation, crosslinking, activation of interpolyelectrolyte complex shells and modification of surface of composite particles. The method offers the opportunity for immobilization of different ligands or bioactive substances, containing amino or hydroxyl groups. Prepared MMPs are nonporous, and this their property minimizes the diffusion factors. High magnetic saturation values of MMPs make them

easy to isolate and concentrate with the use of a laboratory permanent magnet without application of laborious separation methods such as chromatography, filtration and centrifugation. We believe that the techniques described here can be applied for stabilization and functionalization of nanoparticles of metals or their alloys.

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