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Preparation and characterization of magnetic chitosan nanospheres

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A method for the preparation of magnetite/chitosan composite nanoparticles was developed. Colloidal magnetite particles (Fe_3O_4) produced by co-precipitation and stabilized in suspension by adding a non-ionic surfactant (Pluronic F127) were subsequently covered with a layer of chitosan (CS) prepared by ionotropic gelation using sodium tripolyphosphate (STPP) as a crosslinking agent.

The products were characterized in terms of the following parameters: size distribution and ζ-potential (by laser diffraction analysis), surface morphology (TEM), composition (FTIR, elemental analysis), magnetic properties (magnetic susceptibility analysis), and concentration of surface functional groups (potentiometric titration).

The synthesis parameters were optimized for obtaining uniformly distributed colloidally stable, biocompatible magnetic nanoparticles with a high concentration of surface amino groups available for subsequent attachment of biologically active ligands.

Key Words: Chitosan, magnetite, magnetic particles, biocompatible.

Introduction

Magnetic nano and micron sized spheres are widely used in biomedical applications such as diagnostics, magnetic separation, and purification of biomolecules, as contrast enhancement materials in tissue magnetic resonance imaging, carriers for targeted drug delivery, hyperthermia cancer treatment, and in rapid blood detoxification methods.[1] Their unique properties are granted by their high surface area and behavior in the presence of a

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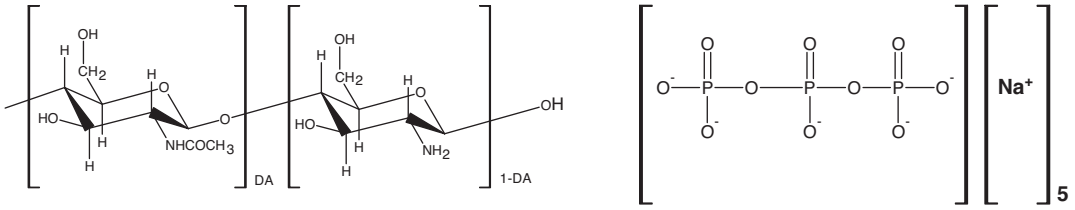
magnetic field. A large number of methods for their preparation have been described in the literature.[2] Due to its biocompatibility, the most common material is iron oxide (magnetite, Fe_3O_4) or its oxidized version, γ-Fe_2O_3 (maghemite), used as a core covered with a matrix or a shell of a natural or synthetic polymer.[3] Among the natural polymers, chitosan is frequently mentioned in recent studies due to its advantageous properties.

Chitosan is derived from the second most abundant natural polymer, chitin (major component of crustacean shells), by partial deacetylation in alkaline conditions. It is a biocompatible, biodegradable, and non-toxic polyaminosaccharide. Its chemical formula (poly(1-4)-2-amino-2-deoxy-D-glucan) is shown in Figure 1a.



(a) (b)

Figure 1. Chemical formulas: 1a. Chitosan (CS); 1b. Sodium tripolyphophate (STPP).

Due to its interesting functional groups (amino and hydroxyl) chitosan is widely used in biomedical applications. Non-magnetic chitosan micro and nano particles for drug delivery applications can be prepared by various methods, such as emulsion crosslinking, coacervation, spray-drying, emulsion droplet coalescence, ionic gelation, reverse micelle preparation, and sieving.[4] Composite chitosan – magnetite nanoparticles intended for drug delivery have been prepared using O-carboxymethylated chitosan,[5] poly(acrylic acid) – chitosan copolymer,[6] or chitosan grafted poly (N-isopropyl acrylamide-co-N,N'-dimethyl acrylamide) copolymer[7] as the encapsulating layer.

Depending on its degree of deacetylation and molecular weight, chitosan is soluble in weakly acidic solutions, in which it shows a polycationic character. Chitosan particles can be obtained by chemical crosslinking using glutaraldehyde.[8–12] The disadvantage of this method is the toxicity of these agents. Ionic gelation (polyionic coacervation), on the other hand, uses non-toxic polyanions, such as STPP (chemical formula shown in Figure 1b).[13–16] In this case the crosslinking mechanism is ionic. The main disadvantage is that soft, pH dependent particles are obtained.[17] In most formulations non-magnetic particles are described. Magnetite – chitosan particles crosslinked with STPP have also been prepared by emulsion crosslinking method, involving laborious washing steps with organic solvents.

This study reports a method for preparing magnetic chitosan nanospheres, optimization of the synthesis parameters, and characterization of the product. Colloidal Fe_3O_4 nanoparticles prepared by direct precipitation and stabilized with surfactant were subsequently covered with a layer of chitosan deposited by ionic gelation using STPP as a crosslinker. The method is simple and reproducible and uses non-toxic reagents.

Experimental

Materials

Iron(II) chloride tetrahydrate ($FeCl_2.4H_20$) and iron(III) chloride hexahydrate ($FeCl_3.6H_20$) were purchased from Merck KGaA. Five different kinds of chitosan were used in this study: low molecular weight (LMW),

medium molecular weight (MWM), and high molecular weight (HMW) procured from Sigma-Aldrich; and CHV2 and CHV3 from Vanson Company. Sodium tripolyphosphate (STPP) 85% and Pluronic F127 were also purchased from Sigma-Aldrich. Sodium hydroxide (pellets, 97%) was purchased from Fluka. Glacial acetic acid was received from Chemical Company. All solutions were prepared with distilled water.

Magnetite particles preparation

Magnetite (Fe_3O_4) nanoparticles were prepared by co-precipitation using Pluronic F127 as a non-ionic surfactant. Iron(III) chloride hexahydrate (0.0551 mol) dissolved in 84 mL of water mixed with 36 mL of aqueous solution of Pluronic F127 (2%) and iron(II) chloride tetrahydrate (0.0275 mol) dissolved in 84 mL of water mixed with 36 mL of 2% surfactant solution were mixed in a 500 mL 3-necked flask equipped with mechanical overhead stirring placed in a temperature controlled water bath. A sodium hydroxide aqueous solution (12.8 g in 120 mL of distilled water) was then added using a peristaltic pump with flow rate of 10 mL/min while stirring at 65 °C. The reaction was continued for 30 min under the same conditions. The resulting Fe_3O_4 particles were washed repeatedly with distilled water until neutral pH using magnetic field separation. The solid content (w/w) was determined for the final suspension by drying an aliquot.

Preparation of composite particles

The magnetite particles obtained in the previous step were covered with a layer of chitosan by ionotropic gelation using the same experimental set-up. The recipe below describes the amounts used in the synthesis of lot NMC2 (optimum parameters). The parameters used for preparing the other lots are shown in Table 1. The STPP solution was used either as it is (0.15% STPP in distilled water shows pH=9.6) or adjusted at pH=6.5 and pH=3 respectively using 1 M HCl.

The magnetite suspension (12 mL, solid content 1.245% w/w) was first mixed with 11 mL of surfactant solution and re-dispersed using an ultrasonic bath for 5 min, and then placed in the flask. After that 75 mL of chitosan solution (0.2% chitosan CHV3 in 0.5% acetic acid) was added and the reaction mixture was stirred for half an hour. Freshly prepared crosslinker solution (140 mL of aqueous STPP, 0.15% w/w, pH=9.6) was then added using the peristaltic pump (10 mL/min). After continuing the reaction for 1 h at room temperature, the mixture was placed in the fridge and left to mature overnight. The resulting particles were magnetically separated the next day and washed 4 times with 500 mL of distilled water each. After the final wash, the suspension volume was adjusted to 50 mL.

Characterization

Particle size distribution was determined on a laser diffraction analyzer (Shimadzu SALDI-7001). The surface morphology was evaluated by TEM microscopy (Philips CM 20 apparatus operating at 100 keV) performed on a sample that was deposited as wet dispersion on the grid and then air dried before being loaded into the microscope. The magnetization was measured on a VSM 7410 vibrating sample magnetometer. Chemical composition was analyzed by FTIR (FTIR Bomem MB 104 spectrometer) and elemental analysis. Thermogravimetric analysis was performed on a Mettler Toledo TGA-SDTA851 system, in nitrogen atmosphere, at a

heating rate of 10 K/min and sample size of 4-6 mg. Particle ζ-potential was measured in phosphate buffer (pH=6) on a Malvern Zetasizer ZS90 system.

Table 1. Synthesis parameter study.

Batch	CS CS MW	Mag/CS ratio, w/w	pH STPP	CS conc, %	STPP conc, %	Temperature, °C	Average diameter, μm
NMC1	94,800	2	9.6	0.2	0.15	19	1.6
NMC2	**94,800**	**1**	**9.6**	**0.2**	**0.15**	**19**	**0.9**
NMC3	94,800	0.5	9.6	0.2	0.15	19	20.5
NMC4	94,800	1	3.0	0.2	0.15	20	36.5
NMC5	94,800	1	6.5	0.2	0.15	20	19.6
NMC6	94,800	1	9.6	0.2	0.3	20	6.1
NMC7	94,800	1	9.6	0.2	0.075	20	11.1
NMC2_B	**94,800**	**1**	**9.6**	**0.2**	**0.15**	**24**	**0.6**
NMC2_C	**94,800**	**1**	**9.6**	**0.2**	**0.15**	**26**	**0.9**
NMC2_F	**94,800**	**1**	**9.6**	**0.2**	**0.15**	**23**	**0.39**
NMC2_G	**94,800**	**1**	**9.6**	**0.2**	**0.15**	**23**	**0.33**
NMC8	94,800	1	9.6	0.1	0.15	19	16
NMC9	94,800	1	9.6	0.2	0.15	40	1.7-2.3
NMC10	415,200	1	9.6	0.2	0.15	23	64.8
NMC11	94,800	1	9.6	0.2	0.15	15	60
NMC13	LMW[1]	1	9.6	0.2	0.15	23	0.33
NMC14	MMW[2]	1	9.6	0.2	0.15	23	1.1
NMC16	HMW[3]	1	9.6	0.2	0.15	23	0.9

[1]Low molecular weight chitosan (Sigma-Aldrich), MW = 50,000-190,000

[2]Medium molecular weight chitosan (Sigma-Aldrich), MW = 190,000-300,000

[3]High molecular weight chitosan (Sigma-Aldrich), MW = 310,000-375,000

The concentration of the surface amino groups was determined by conductometric titration, using a concentrated particle suspension (0.4449 g in 20 mL), with pH adjusted to 6.7. The suspension was titrated with 0.005 M HCl, while monitoring the conductivity with a Consort C831 analyzer.

Results and discussion

Magnetite particles preparation

Colloidal magnetite particles (Fe_3O_4) were prepared by direct co-precipitation from an aqueous solution containing Fe^{3+}/Fe^{2+} ions with a molar ratio of 2:1, upon addition of aqueous sodium hydroxide, according to the chemical reaction shown below:

$$Fe^{2+} + 2Fe^{3+} + 8H_2O => Fe_3O_4 + 4H_2O$$

It is known that magnetic particles tend to agglomerate spontaneously and lose magnetization due to an oxidation reaction. Using a surfactant during synthesis avoids these possible negative effects. Pluronic F127 (poloxamer 407) is a non-ionic surfactant composed of a tri-block copolymer with a hydrophobic poly(propylene oxide) core and hydrophilic poly(ethylene oxide) side chains:

$$HO(C_2H_4O)_{101}(C_3H_6O)_{56}(C_2H_4O)_{101}H$$

The core attaches easily to the weakly hydrophobic surface of magnetite particles, while the side chains provide colloidal stability, hydrophilic character, and biocompatibility.[18]

Two lots of magnetite were synthesized. The average diameter of the particles was 24 nm in the first one and 25 nm in the second one, proving that the synthesis method was reproducible. The colloidal suspension was stable over a 6-month period.

Composite particles preparation

The magnetic particles obtained in the previous step were subsequently covered with a layer of chitosan (CS) prepared by ionotropic gelation using sodium tripolyphosphate (STPP) as a crosslinking agent. The chitosan types used in this study had the average viscosimetric molecular weight between 50,000 and 214,000 and the deacetylation degree in the range of 75% to 85%.

Synthesis parameters optimization

The following synthesis parameters were studied: molecular weight of CS, magnetite/CS ratio, STPP solution concentration and pH, concentration of the chitosan solution, and the temperature during crosslinking reaction. The results are presented in Table 1.

The average viscosimetric molecular weight of the chitosan types is shown in the second column. In the last column the average particle diameter is displayed, as it resulted from the number size distribution in the laser diffraction measurement.

The synthesis parameters were optimized to yield the lowest average diameter of the resulting particles. Lots NMC2, NMC2B, C, and F are synthesized under the same conditions (optimum) and resulted in particles with comparable size (between 0.3 and 0.9 μm), proving that the process is reproducible. These are shown in the table in bold characters. Lot NMC2 was fully characterized and the results are reported in the analysis section.

Overlay size distribution curves of lots NMC2, NMC2B, and NMC2C are shown in Figure 2.

The results also prove that the particle average diameter decreases with increasing chitosan molecular weight. Two chitosan types with comparable molecular weight (Vanson CHV3, MW=94,800 and LMW from Sigma-Aldrich, MW in the range of 50,000 to 190,000) yielded particles with similar size (lots in the NMC2 series compared with lot NMC13). Lots NMC2, 5, and 4 were synthesized with STPP solutions with pH of 9.6, 6.5, and 3, respectively. The particle diameter increased linearly when decreasing the pH, as shown in Figure 3.

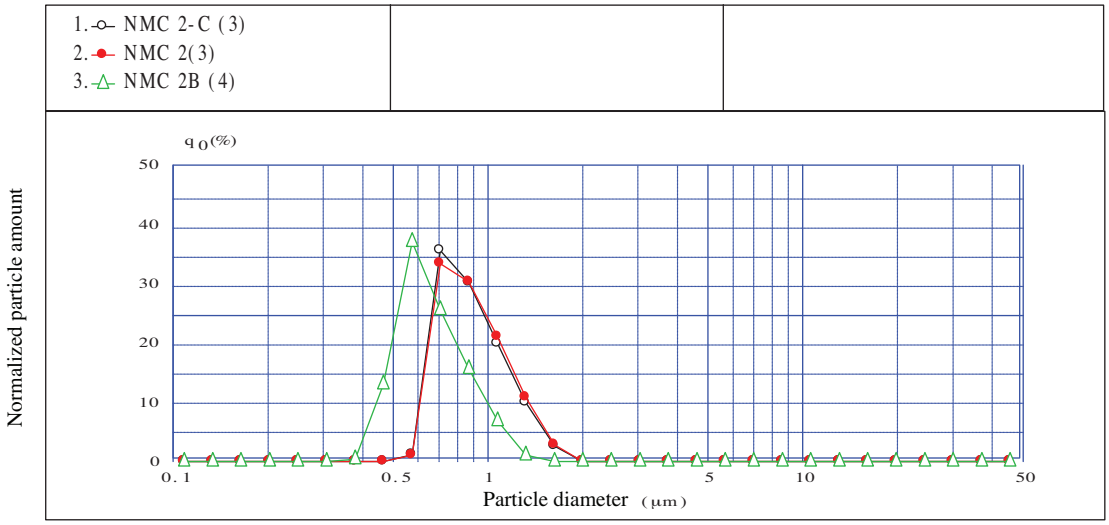


Figure 2. Particle size distribution for 3 lots synthesized under the same experimental conditions.

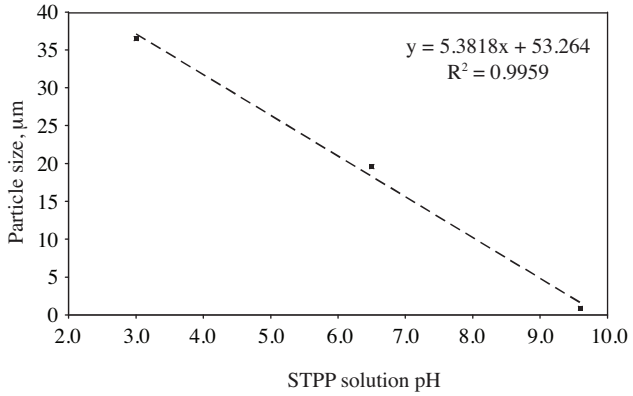


Figure 3. Particle diameter versus pH of STPP solution.

Higher pH values of the STPP solution favor ionic interaction between the OH^- groups belonging to the crosslinker and the NH_2^+ groups belonging to the chitosan macromolecules dissolved in aqueous acetic acid. This process leads to formation of a densely packed structure yielding smaller particles.

Magnetic properties

The magnetization curves obtained for bare magnetite and composite particles are presented in Figure 4.

The magnetic saturation was 61.2 emu/g for bare magnetite and 29.6 emu/g for CS/magnetite particles, which results in estimated magnetite content of 50% in the composite particles. Given the fact that the particles were prepared using a 1/1 CS/Magnetite ratio we can conclude that all the magnetite was incorporated.

FTIR spectroscopy

Overlay FTIR spectra of raw chitosan (Vanson CHV3), Pluronic F127, bare magnetite, and composite particles (lot NMC2) are shown in Figure 5.

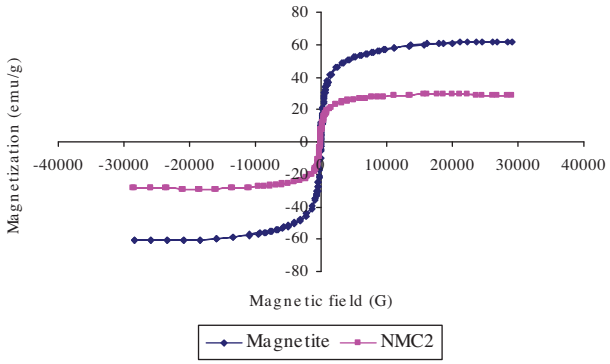


Figure 4. Magnetization versus magnetic field for bare magnetite and composite particles (lot NMC2).

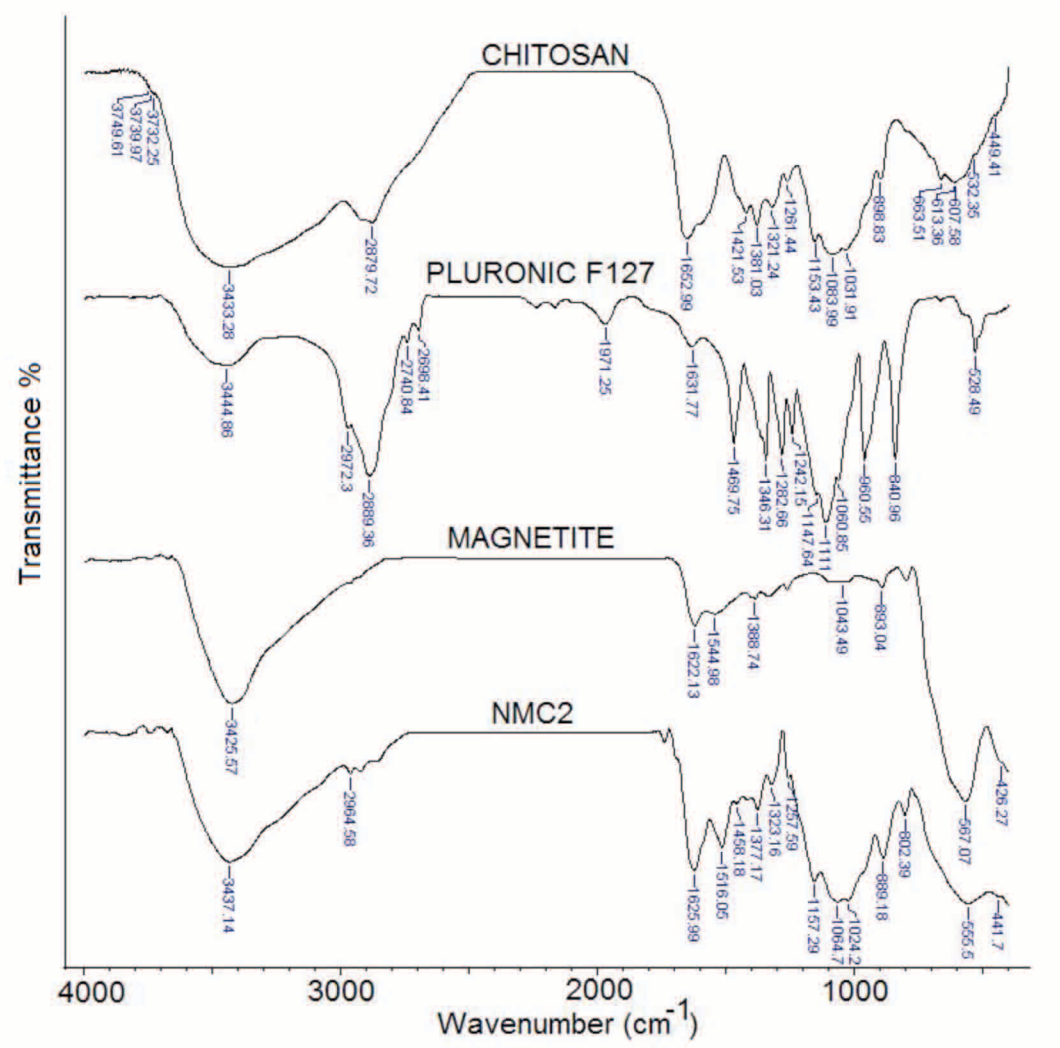


Figure 5. FTIR spectra of chitosan (Vanson CHV3), surfactant (Pluronic F127), bare magnetite, and composite particles (lot NMC2).

The peak located in the 560 cm^{-1} region, characteristic for the Fe-O group, is found in raw magnetite and composite particles spectra, confirming that the product contains magnetite. The peak around 1650 cm^{-1}, assigned to the N-H group bending vibration, is present both in raw chitosan and composite material spectra, proving that magnetite particles were successfully covered with polymer. Due to crosslinking, its appearance is slightly modified in the NMC2 spectrum.

Elemental analysis

Comparative results of elemental analysis of bare magnetite and composite particles are presented in Table 2.

Table 2. Elemental analysis.

Sample	Nitrogen (%)	Carbon (%)	Hydrogen (%)	Residue (%)
Magnetite	0.1	11.2	-	84.9
NMC2	1.875	29.36	2.77	53

The increase in nitrogen and carbon content and the decrease in residue percentage confirm that a chitosan layer was deposited successfully on the magnetite support. From these results it can be approximated that the particles contain about 45% magnetite by weight, which is in good agreement with the estimate from the magnetization measurement. It also proves that most of the magnetite used was incorporated in the particles (CS/Magnetite = 1/1 in the recipe).

Thermogravimetric analysis

The results of the thermogravimetric analysis are presented in Table 3. For each sample (Pluronic F127, Vanson CH3 chitosan, raw magnetite, blank and composite particles) the peak temperature in each degradation step and its percentage weight loss are shown in the third and fourth columns, respectively. The blank sample is prepared in the same way as the composite particles, but without adding colloidal magnetite, and consists of crosslinked chitosan particles that have no magnetic core.

Differential thermogravimetric curves of the same samples are presented in Figure 6.

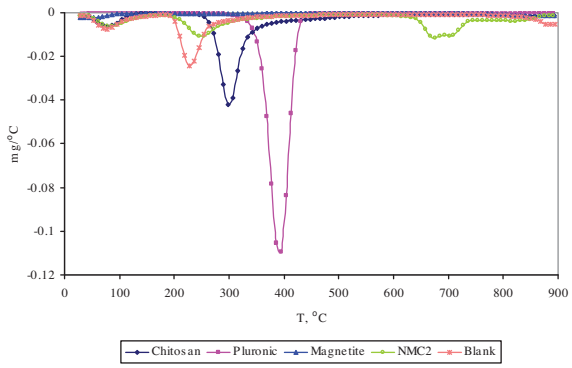


Figure 6. DTG curves of chitosan, surfactant (Pluronic F127), bare magnetite, composite particles (lot NMC2), and blank (crosslinked chitosan particles without magnetite).

Table 3. Thermogravimetric analysis.

Sample	Decomposition step	T_{peak},∘C	W%
Pluronic	I	392	98.65
Chitosan	I	66	7.50
	II	318	65.15
Magnetite	I	56	2.67
	II	288	1.48
	III	862	3.60
Blank (crosslinked chitosan, no magnetite	I	76	11.51
	II	228	38.23
	III	-	10.39
NMC2	I	84	9.46
	II	246	20.34
	III	672	18.59
	IV	838	8.74

The results show that thermal decomposition of Pluronic F127 occurs in one step at 392 °C. All the other samples eliminate water in the range of 56 to 84 °C. Raw magnetite prepared using surfactant has a hydrophilic character and it shows very little weight loss when heated up to 900 °C. The second decomposition step in raw chitosan occurs at 318 °C. By comparison, composite particles of lot NMC2 show a second step at lower temperature, 246 °C, probably due to the fact that deposited chitosan has lower crystallinity than the raw material. The third decomposition step in the NMC2 sample, occurring at 672 °C, is probably related to the decomposition of a fraction composed of highly crosslinked chitosan. Given the fact that this step does not occur in the blank sample, we conclude that the formation of a denser crosslinked network is favored by chitosan molecules adsorbed on the surface of magnetite colloid rather than the ones moving freely in solution, as was the case when the blank was prepared.

Microscopy

The particle morphology was analyzed by TEM (bare magnetite and composite particles) and the images are presented in Figures 7 and 8.

The images show that the composite particles consist of multiple magnetite nanoparticles incorporated in chitosan gel. The detail at higher magnification in Figure 8 confirms that the magnetite nanospheres remain distinct, keeping their colloidal stability. They do not aggregate inside the cluster to the point where they would cancel their individual magnetic moments. This explains the high magnetization of the product.

Surface properties

The results of ζ-potential measurement and of the conductometric titration are presented in Table 4.

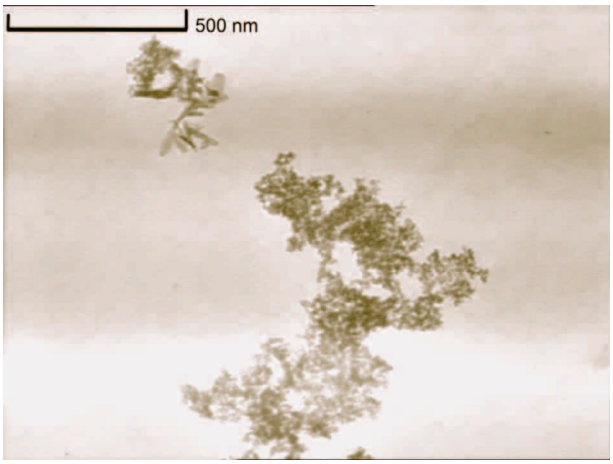


Figure 7. TEM image of bare magnetite particles.

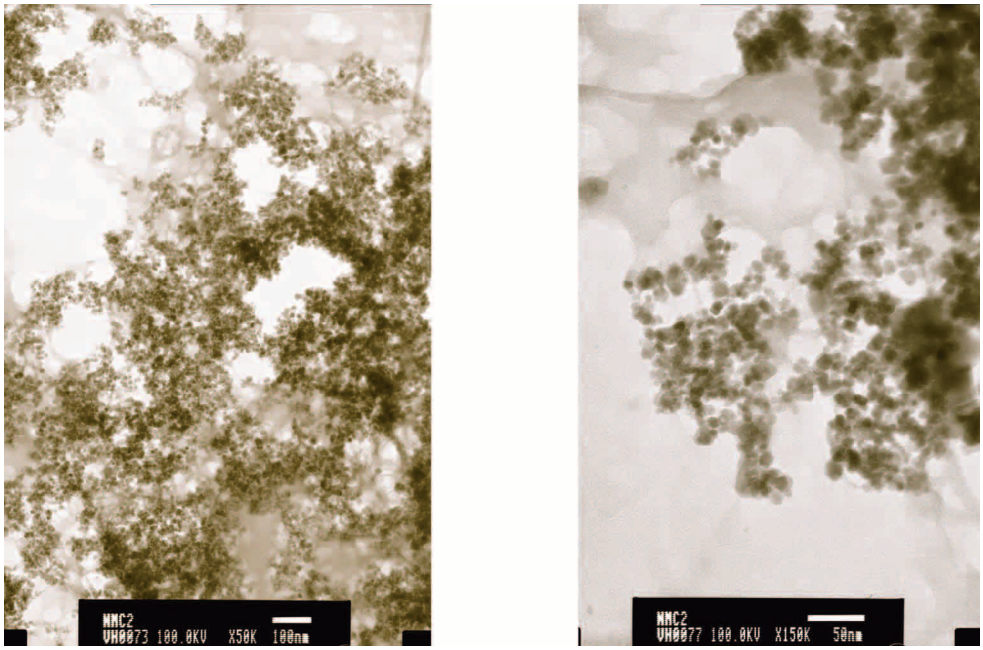


Figure 8. TEM images of composite particles (lot NMC2).

Table 4. Surface properties.

	$\zeta-$potential, mV	NH_2 groups equiv/g
Magnetite particles	- 12.7	-
NMC2	+ 0.974	6.7×10^{-5}

$\zeta-$potential measured in 0.2 M phosphate buffer, pH = 6.

Surface amino groups determined by conductometric titration of a concentrated suspension of pH = 6.7.

Magnetite particles are negatively charged due the presence of adsorbed hydroxyl ions. After chitosan layer deposition they become positively charged. The ζ-potential measurement was performed at pH=6. All the amino groups belonging to chitosan are protonated at pH lower than 6.5. The concentration of the surface amino groups was estimated by conductometric titration and the curve is presented in Figure 9.

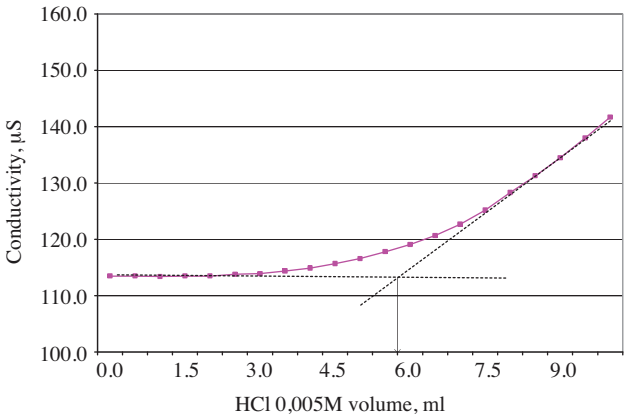


Figure 9. Conductometric titration curve.

As hydrochloric acid is added to the sample (all the amino groups belonging to chitosan are in NH_2 form at pH=6.7, at the beginning of titration), it reacts with the surface NH_2 groups and the conductivity remains constant. After protonating all the amino groups, any excess acid causes an increase in conductivity.

The results prove that composite particles bear surface free amino groups available for functionalization with biologically active ligands.

Conclusions

A simple method for preparation of magnetite/chitosan composite nanoparticles using co-precipitation followed by ionic gelation was developed. The synthesis parameters were optimized for minimum particle diameter. Composite magnetite-chitosan particles with an average diameter of 0.6 to 0.9 μm were obtained in a reproducible manner. The particles were characterized with respect to size, morphology, magnetic behavior, and concentration of surface functional groups. The product can be used in bio-separation methods after attaching an application specific ligand to the surface amino groups.

Acknowledgements

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