Magnetic polymer particles prepared by double crosslinking in reverse emulsion with potential biomedical applications

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This study was performed in order to obtain polymeric magnetic microparticles based on chitosan, poly(vinyl alcohol) and maghemite as colloidal magnetic nanoparticles. For preparation of particles we used double crosslinking in reverse emulsion procedure. Sodium sulphate (Na_2SO_4) solution is added for ionic crosslinking of the polymers and glutaraldehyde is used as covalent crosslinking agent. The following preparation parameters were studied: concentration of polymers solution, concentration of surfactants, poly(vinyl alcohol) to chitosan weight ratio, stirring speed, molar ratio between crosslinking agents, polymers to magnetic material weight ratio. The characterization of microparticles was performed by Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy, Transmission Electron Microscopy, Thermal Gravimetric Analysis and Vibrating Sample Magnetometry analysis. The evaluations of their toxicity together with hemocompatibility tests have shown that these particles exhibit the prerequisite behavior for use in biomedical field, for drug targeting. Also, was studied the behavior of particles in aqueous media, the drug (5-fluorouracil) loading and the kinetic of drug release from the particles, determined that particles behave differently depending on their structural characteristics, composition and morphology.

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1. Introduction

The materials with physical properties that can be influenced by application of an external magnetic field belong to a specific class of smart materials. Usually, the magnetically responsive materials are formed by magnetic iron oxides (magnetite or maghemite) dispersed in a biopolymer matrix, or attached on the surface of particles, biological structures or cells. Such materials have potential applications within the life science disciplines, biotechnology, biomedicine and environmental technology. Different kinds of polymers can be used for the stabilization and encapsulation of magnetic particles, as well as drugs or cells.

Chitosan is a natural hydrophilic polysaccharide, obtained by alkaline deacetylation of chitin [1]. Chitosan is a biodegradabil, nontoxic and biocompatible biopolymer and these properties make it suitable for use in biomedical and pharmaceutical applications [2]. For different applications, chitosan has been formulated as powder, gels and films, sponges, intragastric floating tablets and micro- and nanoparticles [1, 3, 4]. Methods, such as the emulsion crosslinking [5], ionotropic gelation method [6], reverse micellar method [7], self-assembling method [8, 9], coacervation/precipitation technique [10-12], emulsion-droplet coalescence [13], template polymerization [14] have been used to prepare chitosan micro/nanoparticles. Also, the preparation of magnetic polymer particles based on chitosan was already reported in literature [15-17].

Poly(vinyl alcohol) is a synthetic polymer, water-soluble, odorless and nontoxic and has very good emulsifying and adhesive properties, usually is used as the stabilizer [18]. Poly(vinyl alcohol) containing large amounts of hydroxyl groups has been developed for biomedical applications since it is biocompatible and nontoxic, and exhibits minimal cell adhesion [19].

These two polymers have previously been successfully used in the preparation of non-magnetic polymer particles by using the same preparation method (double crosslinking in reverse emulsion method) [20], which led us to the idea of preparing magnetic polymer particles based on chitosan and poly(vinyl alcohol). This double crosslinking in reverse emulsion method was reported already for preparation of polymer particles obtained from two different polymers [21, 22].

The main aim of this study is the preparation of magnetic microparticles based on chitosan and poly(vinyl alcohol) mixtures of interpenetrated/interconnected network type (IPN), prepared by a two-step crosslinking technique applied in reverse emulsion system. The first step is the ionic crosslinking realized by using nontoxic crosslinking agents like sodium sulfate solution. In the

second step, a low amount of covalent crosslinking (glutaraldehyde) was used to stabilize the shape and dimensions of the obtained particles. Considering the fact that magnetic particles can be directed to the disease site in cancer therapy, the particles were loaded with 5-fluorouracil. 5-fluorouracil is an anticancer drug with a broad spectrum, used usually for treatment of breast cancer, head and neck cancers, anal cancer, stomach cancer, colon cancer and some skin cancers. The study was orientated to the morphology optimization of the magnetic polymer microparticles, through the variation of preparation parameters.

2. Experimental

2.1 Materials

Chitosan low molecular weight (CS) (91.1% deacetylation degree), Poly(vinyl alcohol) (PVA) (Mw 9,000 – 10,000, 80% hydrolyzed), Toluene, Acetone, Sodium sulphate (10% aqueous solution), Glutaraldehyde (25% aqueous solution) for synthesis (GA), n-hexane, Span® 80 and Tween® 80, 5-fluorouracil (5-FU) were purchased from Merck, Germany. Glutaraldehyde was extracted from toluene and then was used as crosslinker during the preparation process of the polymer magnetic particles. Glacial acetic acid p.a. grade was received from Chemical Company, Romania. All aqueous solutions were prepared using double distilled water. All other chemicals used in this work were of analytical grade purity and used without further purification.

2.2 Methods

2.2.1 Preparation of maghemite:

The cationic ferrofluid containing maghemite was prepared in our laboratory according to Massart method [23]. The nanoparticles of magnetite were obtained by coprecipitation of ferric and ferrous salts in ammonium medium (Fe^{3+}/Fe^{2+} molar ratio of 1.5) which was oxidized to maghemite, at 80°C with iron (III) nitrate, followed by peptization process with nitric acid. The final product has the pH environ 2.5, the maghemite concentration in ferrofluid is 9.65 % (w) and the average particle diameter is 44.3 nm.

2.2.2 Preparation of chitosan-PVA magnetic microparticles:

Magnetic chitosan-PVA microparticles were prepared by water/oil (w/o) emulsion crosslinking method using two different crosslinking agents, an ionic crossliker (sodium sulphate) and a covalent one (glutaraldehyde).

Briefly, the polymer solution (different concentrations) was prepared by dissolving chitosan and PVA in acetic acid solution 2% by continuously stirring at 80°C and then stirred overnight until the complete homogenization. In this solution the ferrofluid was added by dropping. Then, different concentrations of Tween 80 (according with experimental plan) were added as hydrophilic stabilizing agent. This solution, which is the aqueous phase, was dropped in the organic phase (fourth times more than volume of aqueous phase), composed by toluene with Span 80 (as hydrophobic tensioactive agent, different concentrations), by continuously stirring under high speed stirring. Then, the ionic crosslinker, sodium sulphate solution, was dropped in the w/o emulsion with a syringe with sharp needle. After 10 minutes of ionic crosslinking, the mixture was moved in a glass reactor equipped with a mechanical stirrer. Under vigorous stirring, glutaraldehyde extracted toluene, as covalent crosslinker, was added drop by drop and the covalent crosslinking reaction was carried out for 6 hours at room temperature at 500 rpm stirring speed. When the reticulation time was completed, the particles were separated from emulsion by centrifugation and washed repeatedly with acetone, water and with n-hexane to remove the toluene, the surfactants, the non reacted polymers and excess of the crosslinking agents. The microparticles were dried from n-hexane at room temperature. The following preparation parameters were studied: polymers solution concentration (0.3, 0.5, 1 %), surfactants concentration (2, 3, 4%), PVA–CS weight ratio (1/9, 3/7, 5/5 and 7/3 w/w ratio), rpm stirring speed (6000, 9000, 12000), crosslinking agents molar ratio (1/2, 1/1, 3/2), polymers-maghemite weight ratio (4.5, 2.25, 1.5).

3. Characterization

3.1 Fourier transforms infrared spectral measurements

Fourier transform infrared (FTIR) spectroscopy was used to characterize the magnetic polymer microparticles. The FTIR spectra were recorded for CS, PVA and magnetic polymer particles, using a Thermo Nicolet Nexus FTIR spectrophotometer, to confirm the formation of ionic and covalent bonds and to demonstrate the presence of the maghemite into the prepared particles. FTIR spectra of plain CS, plain PVA, plain maghemite and polymer particles based on chitosan and PVA and magnetic polymer particles based on chitosan and PVA were obtained under identical conditions. The samples were ground with KBr and pressed into pellets for FTIR transmission measurements. Spectral scanning was done from 4000 to 400 cm^{-1}.

3.2 Thermogravimetric analysis

The maghemite content of the magnetic polymer microparticles was determined by thermogravimetric analysis (TGA), using a SDT-Q600 derivatrograph, under nitrogen atmosphere characterized by a 100 ml/min flow, with a thermobalance between 25 and 900°C, at a temperature rate of 20°C/min. The samples weighed 8 – 10 mg. In order to get comparable data, the operation parameters were kept constant for all the samples. Curve

processing designed to determine the thermal and kinetic characteristics was done using the software developed by SDT-Q600. The thermal tests were accomplished for chitosan, PVA, maghemite, polymer particles and magnetic polymer microparticles based on CS and PVA, in order to calculate the γ-Fe_2O_3 content.

3.3 Nitrogen determination

The Kjeldahl analysis was used to determine the nitrogen content from chitosan and for magnetic polymer particles based on CS and PVA. Based on nitrogen content, we calculated the chitosan content in particles. The results from chitosan content and those from TGA analysis allow to determine the composition of magnetic polymer particles.

3.4 Magnetic properties

The magnetic properties of the magnetic material and magnetic polymers microparticles were obtained by vibrating sample magnetometer (MicroMag, VSM - Vibrating Sample Magnetometer, Model 3900, Princeton Measurements Corporation, USA), at room temperature.

3.5 Scanning Electron Microscopy

The shape, size and surface morphology of the polymer magnetic microparticles were investigated with field-emission scanning electron microscope (SEM) JSM-7401F, JEOL (Tokyo, Japan) at an accelerating voltage of 4.0 kV, at different magnification (20.000-30.000 times). For the sample preparation, the polymer magnetic particles (dry, powder) were spread in very thin layer on tape support covered with carbon. The particle layers were coated by gold which was carried out by the Sputter coater from BALTEC SCD 050, at pressure of 0,05 mbar, sputtering current was 56 mA, 50 nm thickness of gold for 80 s.

3.6 Transmission Electron Microscopy

Information on the intraparticle morphology of the magnetic polymer microparticles was performed with transmission electron microscopy (TEM) system.

The polymer magnetic particles (dry, powder) were mixed with resin EPON in Eppendorf tubs, then were centrifuged 1000 rpm for 5 min, after that were polymerized at 63°C in a Biological thermostat BT 120 for 48 h. After 2 days the samples were cut in ultra-thin sections (90 nm) on LEICA ultramicrotome and observed by transmission electron microscope JEOL 1010 (Tokyo, Japan) at 80 kV.

3.7 Particle size and size distribution

The average particle size and size distribution of the magnetic polymer particles were measured by laser diffraction analysis using a Shimadzu SALD-7001 particle size analyzer. The dispersion of magnetic polymer particles in h-hexane was sonicated in an ultrasonic bath for 2 minutes before measurement at room temperature, under constant stirring.

3.8 Particles toxicity

The evaluation of toxic side effects of all substances intended for use in animals and humans body is a “must know” property; also the toxicity for magnetic polymer particles, if they are designated for drug targeting in cancer therapy. The evaluation of toxicity of the magnetic polymer microparticles was realized by measuring the lethal dose (LD_{50}). The LD_{50} measured the short-term toxic potential (acute toxicity) of materials and is expressed as the weight, in mg, of the material administered per kilogram body weight of the animal (mg/kg body). The LD_{50} represents the amount of a substance, administered all at once, which kill 50% (one half) of animals tested. The magnetic polymer microparticles were administered as a suspension in Tween 80 via the intraperitoneal way on rats weighing 20 ± 2 g, according to the classical laboratory methodology. For the interpretation of the LD_{50} values of the microparticles was used the Hodge-Sterner toxicity scale [24]. According to this toxicity scale, a LD_{50} value under 1 mg/kg body mass is considered extremely toxic, a value between 1 and 50 mg/kg body mass is very toxic, a value between 50 and 500 mg/kg body mass is moderately toxic, a value between 500 and 5000 mg/kg body mass is low toxic, a value between 5000 and 15000 mg/kg body mass is practically nontoxic, and a value above 15000 mg/kg of body mass is considered nontoxic.

3.9 Hemocompatibility tests

For evaluation of hemocompatibiliy is necessary to determine the prothrombin time (PT) and to calculate the international normalized ratio (INR) of blood in contact with nanoparticles. The integral blood of a healthy and non-smoker volunteers was collected by venous puncture. Then, the blood was incubated with an anticoagulant (aqueous sodium citrate 3.8 % w/v; ratio 1/9 v/v). After that, in 3 mL blood was added a suspension of nanoparticles in physiological serum (0.5 mL; 0.02 % w/v) to obtain 0.015 mg/mL nanoparticle concentration in blood. The control samples were prepared by added 0.5 mL of physiological serum to the same volume of integral blood. The samples and the controls were incubated at 37°C for 30 min under gentle shaking. Then, the centrifugation at 1000 rpm, for 10 min was used for separation of particles from blood cells. For determination of PT in blood plasma, three values were mediated, using an blood tester (ACL 100) and a PT-Fibrinogen kit (Biodevice, Italy) which has an International Sensitivity Index (ISI) = 1.06.

The INR parameter is calculated as the particles prothrombin times (PT_{nano}) and control samples prothrombin times (PT_C) ratio raised to the power of the ISI value:

$$INR = (PT_{nano} / PT_C)^{ISI} \quad (1)$$

3.10 Swelling experiments

The swelling behavior was studied by measuring the weight of particles immersed in phosphate buffer solution, PBS (pH=7.4). The dry samples were weighted in Eppendorf tubes and then were allowed to swell for 24 hours, to ensure the complete swelling (preliminary tests showed that this is the appropriate time interval). The swollen samples were collected by ultracentrifugation and the excess of liquid was removed by carefully blotting with filter paper. The (dry and swollen) microparticles were weighted to an accuracy of ± 0.0001 g on an electronic microbalance. For calculation of the swelling degree was used the following equation:

$$Q_w(wt\ \%)=[(w-w_0)/w_0]\cdot 100 \quad (2)$$

where w and w_0 are the weight of the swollen and of the dry magnetic polymer microparticles, respectively. Experiments were performed in triplicate, but average values were considered for data treatment and calculations.

3.11 In vitro drug loading studies

The magnetic polymer microparticles already swollen in phosphate buffer solution (pH = 7.4) were suspended in a solution of 5-FU prepared in PBS, with 10 mg/ml concentration, for 24 hours, at room temperature. The using of particles already swollen avoids the competition between water and drug molecules. After 24 hours, the magnetic polymer microparticles were separated from supernatant by ultracentrifugation. The presence of the drug in the supernatant was estimated by Uv-Vis spectroscopy, using a Spectrophotometer NanoDrop ND-1000. That instrument allows the analysis of very low sample volumes, in microliter range. The amount of drug included in respect with the amount of the dried microparticles was determined by the difference between the initial drug concentration and its concentration in supernatant after ultracentrifugation. For calculation of drug included was used a calibration curve of 5-FU at 265 nm wavelength previously performed.

3.12 Drug release kinetics

Drug release was investigated by diffusion through the measurements of the drug concentration in the supernatant after the immersion of the precisely weighed loaded microparticles. This method is based on the utilization of a release medium which is a good solvent for the studied drug and nonsolvent of the microparticles. The release medium was the phosphate buffer solution (pH = 7.4, simulating the pH of the blood). To investigate the drug release, the drug loaded microparticles were first lyophilized. Then, the specific amounts of magnetic polymer microparticles loaded with 5-FU were introduced in the dialysis membranes (12000 Da), immersed in 10 ml PBS and maintained in a shaker at 37°C. The amount of released drug was determined spectrophotometrically at 265 nm.

4. Results and discussions

The functional groups of the both polymers used in this experiment are able to react with the crosslinking agents. Chitosan presents amino groups which react with carbonil groups of the GA forming imine type bonds, and with sulphate groups, forming ionic bonds. PVA presents hydroxyl groups which form with the carbonil groups of the GA either semi-acetal or acetal bonds. The obtained magnetic polymer particles will have a complex structure, of an interpenetrated-interconnected network (IPN). The possible reactions of ionic crosslinking with sodium sulfate and covalent crosslinking with GA are displayed in Fig. 1.

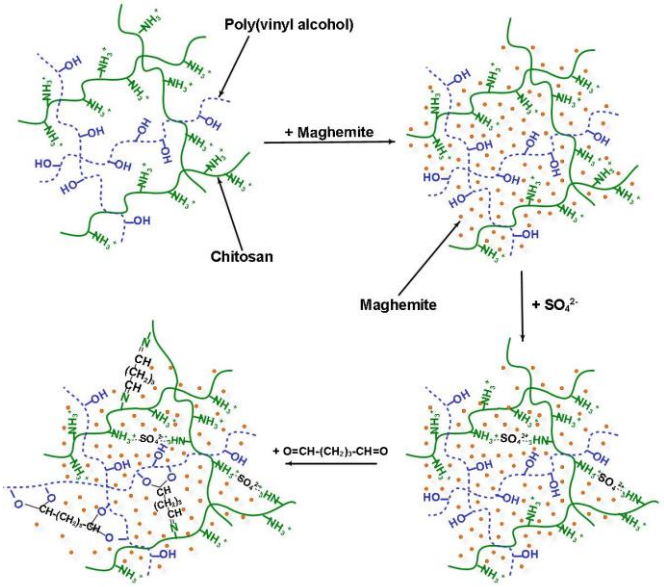

Fig. 1. Ionic and covalent crosslinking reaction between CS and PVA with crosslinking agents, for preparation of magnetic polymer particles.

4.1 Optimization of preparation conditions

The effects of variation preparation conditions on particles properties (morphology, magnetism, drug loading) were investigated and the crosslinking reaction (preparation conditions were) was optimized in order to get the desired product (smaller particles, round in shape, with big amount of magnetic material entrapped). The experiments carried out are listed in (Table 1). It was found that concentration of 0.5% of polymers solution is optimum for obtaining the smaller and compacted particles. When the reaction was carried out with 3% of surfactant, the product obtained is more distinguished and less agglomerated. (The product with 4% surfactant was very difficult to separate and to washed, too.) The PVA: CS ratio of 3:7 w/w was required to obtain the desired product. Since the particles obtained for ratio 1/9 and 3/7 are excellent in shape, we choose to continue the preparation with ratio 3/7 to justify the using of booth polymers, especially because the PVA assures the well defined shape. To diminish the average diameter we grow the speed of agitation from 6000 to 12000, because the biggest is rpm, the smaller are the obtained particles.

The surface of the particles prepared with the lowest amount of sodium sulphate is very harsh, having a sponge-like structure. This aspect becomes less evident while increasing the amount of ionic crosslinker, the particles prepared with equimolar amount of sodium sulphate are more compacte compared with those prepared at ½ or at 3/2 molar ratio.

It was seen that the product with higher polymer maghemite ratio (1.5) was better in terms of magnetization properties but less as morphology; the obtained particles are smaller, but not very compacted and the shape is not really round and not well defined, they are very porous too.

Table 1. The preparation parameters for magnetic polymer particles.

Samples cod	Polymers sol. (%)	Surfactant (%)	PVA/CS (w/w)	rpm speed	SO_4^{2-}/NH_2 (mol/mol)	Polymers/maghemite (w/w)
MCP1	1	2	3/7	6000	3/2	4.5
MCP2	0.5					
MCP3	0.3					
MCP2	0.5	2	3/7	6000	3/2	4.5
MCP4		3				
MCP5		4				
MCP6	0.5	3	1/9	6000	3/2	4.5
MCP4			3/7			
MCP7			5/5			
MCP4	0.5	3	3/7	6000	3/2	4.5
MCP8				9000		
MCP9				12000		
MCP10	0.5	3	3/7	12000	1/2	4.5
MCP11					1/1	
MCP9					3/2	
MCP11	0.5	3	3/7	12000	1/1	4.5
MCP12						2.25
MCP13						1.5

4.2 FTIR

Fourier transform infrared spectroscopy was utilized to confirm the double crosslinking reaction and the presence of magnetic material in microparticles. FTIR spectrum of pure PVA sample reveals the bands: C–H broad alkyl stretching band (2945 cm^{-1}) and typical strong hydroxyl bands for free alcohol (non bonded –OH stretching band at 3435 cm^{-1}). The band at 1444 cm^{-1} is due to CH bending vibration, and the band at 1099 cm^{-1} indicates the stretching of C-O. FTIR spectrum of pure CS sample shows the characteristic band N–H broad (3454 cm^{-1}), O–H stretching vibrations (1639 cm^{-1}), (–C=O) (1056 cm^{-1} (specific for polysaccharide structure). FTIR spectrum of polymer particles prepared from CS and PVA (sample CP) present a new band at 1638 $cm^{-1,}$ indicating the C=N stretching vibration of the imine group of Schiff base. This band confirms the formation of covalent crosslinking between amino groups of the chitosan and carbonyl groups of the glutaraldehyde. The band at 1097 cm^{-1} (attributed to -C-O-C-) proves the reaction of hydroxyl groups of PVA with GA and a new band at 616 cm^{-1} (attributed to SO_4^{2-}) confirms the formation of ionic crosslinking between amino groups of the chitosan and sodium sulphate [22]. The FTIR spectrum of magnetic polymer particles (sample MCP4) presents the same bands as polymer particles (CP). Furthermore, the band at 619 cm^{-1} in MCP4 is larger than the band at 616 cm^{-1} in CP, this is because of the presence of maghemite (the band due to maghemite is overlap band sulphate group/ the band attributed to SO_4^{2-} has been masked by the peak due to the maghemite band in MCP4). These results prove that

maghemite was successfully included in the crosslinked polymeric matrix.

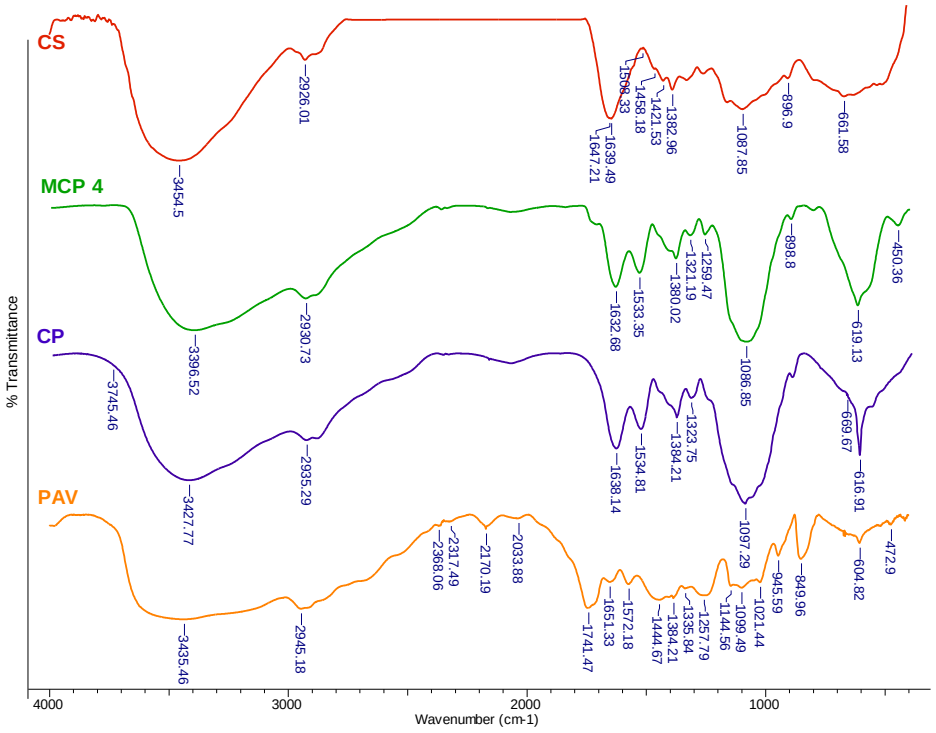

Fig. 2. The spectra of polymers (CS and PVA), polymer particles (CP) and magnetic polymer particles (MCP4).

4.3 Particles size distribution

The average particle size for all the magnetic polymer microparticles are presented in Table 2. The values of the mean diameter are varying from 0.71 to 2.55 μm and strongly depend on preparation parameters. The decrease of polymers concentration determines the decrease of mean diameter particles. The increasing of the concentration of the initial mixture leads to an increase in the viscosity of the polymer aqueous solution. That determine the formation of bigger droplets during emulsification that were later hardened in the presence of crosslinking agents. The increase in the surfactant concentration determines the obtaining of smaller particles. The same observations were made by other authors in their studies [22, 25]. The mean diameter of particles increases with the decrease of PVA/CS weight ratio. As ionic crosslinking takes place only between the sodium sulphate and free amino groups of chitosan (PVA has only hydroxyl groups and it will be crosslinked only by covalent crosslinker, GA) [20], the increase of CS in initial polymer mixture, determines the formation of bigger particles.

PVA has a double contribution in the reaction system. First of all, PVA participate in interpenetrated-interconnected network formation by the crosslinking process induced by glutaraldehyde. Second, it stabilize the polymer-water solution droplets in the organic phase by playing the surfactant role and by protecting the new prepared particles; because of this it is impossible to find it entirely within the particles. As a consequence the yield, after the samples washing and drying, varies between 65-73%.

The same behavior was found for others polymeric systems by [25, 26]. The mean diameter of magnetic polymer particles decreases with the increase of stirring speed. The formulation prepared at 6000 rpm speed in ionic crosslinking step have the medium diameter of 1.74 μm, while formulation prepared at 12000 rpm have the medium diameter of 0.74 μm. A higher stirring speed implies a higher kinetic energy that, transferring over the emulsion, determine the formation of a smaller droplets. A big amount of ionic crosslinker determine a higher crosslinking density so a smaller diameter of particles. Another interesting observation is that particle size decreased with an increase in the amount of maghemite in initial mixture (with the decrease of polymers/maghemite ratio). The increase in maghemite content, determine the decrease in polymer content in particles and that determine the smaller diameter for particles, considering that the network is formed by the crosslinked polymers. The size distribution profiles are presented in Fig. 3. The polydispersity dimensional curves obtained present a monomodal distribution, which is characteristic for one population of particles, in terms of size and the particles have a rather narrow size distribution.

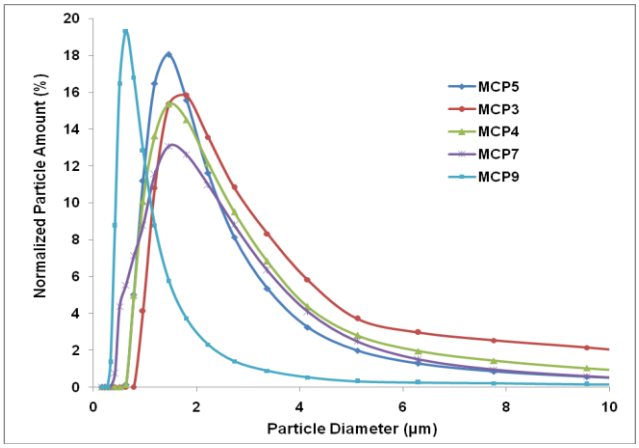

*Fig. 3. Particles size distribution for particles prepared with CS, PVA and maghemite, in different conditions.***SEM**

Fig. 4 reveals the formation of polydispersed particles (sample MCP4). The particles are very well defined spherical shape, the rough surface and the size of the microparticles (ranges from 0.2 µm to 2 µm) is in well accordance with the results obtained from analyzer laser diffractometry and from TEM. SEM micrographs also show that the particle are not very agglomerated (probably, due to the properties of PVA, often used as surfactant). Anyway, the particles can be readily redispersed in water by stirring or sonication. The particles have a relative high dimensional polydispersity as it can be observed in the microscopy images.

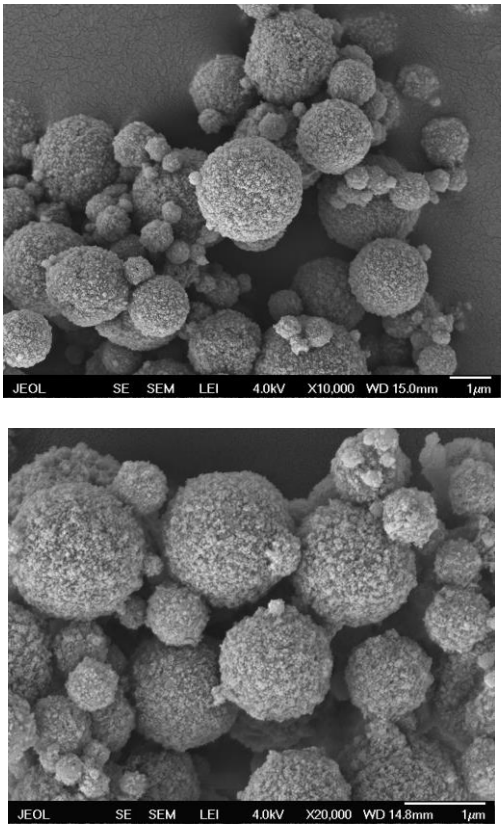

Fig. 4. The SEM microphotographs of magnetic polymer microspheres, sample MCP4 (10000x, 20000x).

4.4 TEM

The TEM images show that the magnetic polymer particles consist of multiple maghemite nanoparticles homogeneously incorporated in crosslinked polymers network (inside and on surface). The detail at higher magnification (Fig. 5) confirms that the maghemite nanoparticles remain distinct, keeping their colloidal stability. They do not aggregate inside the polymer network so they don't cancel their individual magnetic moments. This way can be explained the values of saturation magnetization of the particles reported to the values of maghemite. In the TEM microphotograph, the maghemite nanoparticles can be clearly distinguished: dark black area for the maghemite, and gray area for the crosslinked polymer. The TEM shows that the magnetic nanoparticles are almost present on the surface of the magnetic polymer particles than inside. Even so, inside of the magnetic polymer particle, the maghemite nanoparticles are homogeneous distributed in the crosslinked network (IPN) and also the homogeneous distributions is obvious on particles surfaces. Probably, the rough surface of particles visible in SEM images is due to the presence of the maghemite nanoparticles on surface of crosslinked polymer network (as it can be seen on TEM images).

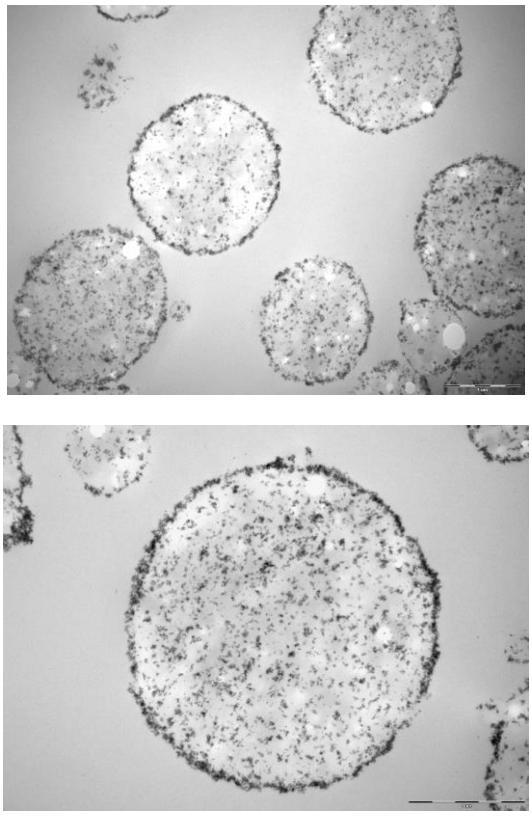

Fig. 5. The TEM micrograph of a magnetic polymer microparticles, sample MCP4 (The scale is 1 micron in both images).

4.5 Thermogravimetric analysis of magnetic polymer particles

The characteristic TGA curves for maghemite and magnetic polymer particles (samples MCP13, MCP12, MCP11) are presented in Fig. 6. The thermograms of maghemite, polymers and particles were obtained at a scan rate of 20°C/min in a nitrogen atmosphere. All prepared particles involve three steps of degradation; the first stage loss of moisture is at low temperature (25-175˚C). In the second stage, the particles begins to decompose at 200˚C, rapidly loses 30-40% of its weight, which could be attributed to degradation of the saccharide rings and disintegration of macromolecule chains of CS. The maximum rate of weight loss for particles occurred between 700˚C and 900˚C.

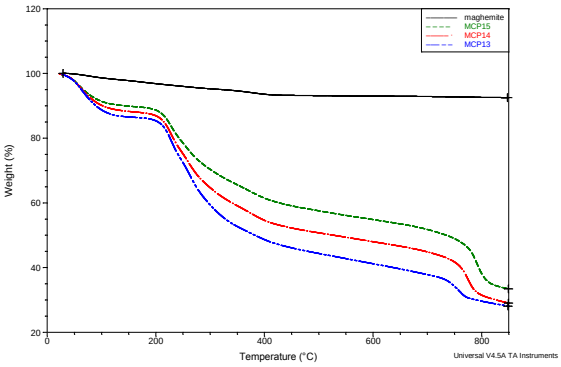

Fig. 6. The termogravimetric curves for maghemite and magnetic polymer particles (samples MCP13, MCP12, MCP11).

The magnetic polymer particles present a major weight loss of 67-76% and the iron oxide from maghemite 7.5%. The CS characteristics played a major role in the thermograms being the major component of the magnetic polymer particles. From the percentage weight loss in the TGA curve, the amount of maghemite from magnetic polymer particles was estimated.

Fig. 7. The magnetic polymer particles suspension in acetone, in absence (first image) and in presence (second image) of magnetic external field (produced by high force magnets).

The average mass content of maghemite in particles by TGA was found to be situated in the range 28% (for MCP13, prepared with the biggest amount of maghemite) and 12.25% (for MCP6, prepared with biggest amount of CS). The magnetic content in the prepared particles is sufficient to be a very good magnetic sensitive in an external magnetic field (generated by a magnet with high force, see the Fig. 7) and can be separated quickly from the separation medium (acetone). This is the reason why the produced magnetic polymer particles can be used for anticancer drug targeting.

Table 2. The final composition, the average diameter, the swelling degree (Q), the amount of drug loaded and the release efficiency of magnetic polymer particles.

Samples cod	Average diameter (μm)	Q (%)	mg 5-FU/g particles	Release efficiency (%)	CS (%)	Maghemite (%)
MCP1	2.55	1207	104.48	75	68.14	13.71
MCP2	2.3	1134	99.58	78	68.07	13.14
MCP3	2.21	1074	105.03	81	67.35	13.23
MCP2	2.3	1134	99.58	78	68.07	13.14
MCP4	1.74	1098	99.37	88	67.4	14.09
MCP5	1.58	1023	93.15	85	67.5	12.82
MCP6	1.9	1347	110.72	94	73.1	12.25
MCP4	1.74	1098	99.37	81	67.4	14.09
MCP7	1.47	950	90.11	87	61.3	15.30
MCP4	1.74	1098	99.37	81	67.8	14.09
MCP8	1.15	1049	97.68	92	67.6	13.75
MCP9	0.741	807	89.01	78	67.5	13.77
MCP10	1.017	1188	100.65	90	69.3	13.02
MCP11	0.913	1067	96.17	80	69.2	15.46
MCP9	0.741	807	89.01	78	67.5	13.77
MCP11	0.913	1067	96.17	80	69.2	15.46
MCP12	0.851	822	89.24	76	68.1	22.12
MCP13	0.717	726	84.25	70	65.2	28.05

4.6 Magnetic properties

Fig. 8 shows the magnetization curve and saturation magnetization (M_s) for magnetic material used and for magnetic polymer microparticles (samples MCP7, MCP12 and MCP13). The magnetization curve passes through the origin of coordonate system. As the increasing and decreasing magnetization curves are mirror images, no values of remanence and coercivity could be deduced from this figure. The absence of hysteresis (M_r=0 and H_c=0), when magnetic field applied, showed that the magnetic polymer microparticles present superparamagnetic properties.

The particles with superparamagnetic properties are attracted to a magnetic field and they retain no residual magnetism when the magnetic field is removed. That properties is critical for the applications in biomedical and bioengineering fields, because prevents microparticles from aggregation and enables them to redisperse rapidly when the magnetic field is removed [27]. It can be seen that the three types of magnetic polymer particles and the maghemite exhibit similar overall magnetic behavior, characteristic of soft magnetic materials (particles), with a narrow hysteresis cycle [28]

The magnetization of saturation (Ms) values for magnetic polymer particles sample MCP11 is 12.75 emu/g, for MCP12 is 16.18 emu/g and for sample MCP13 is 21.43 emu/g, while for maghemite is 48.48 emu/g. The saturation magnetization of MCP13 is somewhat higher than the reported values of samples MCP12 or MCP11 respectively according with the amount of magnetic material used for preparation (initial composition) and especially according with the percent of ferric oxides in particles (final composition, determined by TGA).

4.7 Hemocompatibility tests

These kind of magnetic polymer particles are designated to medical applications, to treatment of tumor diseases, so they will be in contact with the blood, inside the body. All the materials which enter in contact with blood need to be hemocompatibility tested. The interaction of magnetic polymer particles with the components of the blood is evidenced by a change of plasma coagulation properties on incubation with these particles [29]. The effect of magnetic polymer particles on blood coagulation was evaluated with a standard clinical coagulation assay. For to evaluation the extrinsic and common coagulation pathways, the activity of each of five different blood clotting factors (I, II, V, VII and X) was detected and measured. The results of hemocompatibility tests were expressed as the IQ and INR [30].

The values obtained for coagulation times and INR (Table 3) and those for blood sample are located in the normal range for biomaterials are comparable and very close, suggesting that the magnetic polymer particles do not inhibit prothrombin activity.

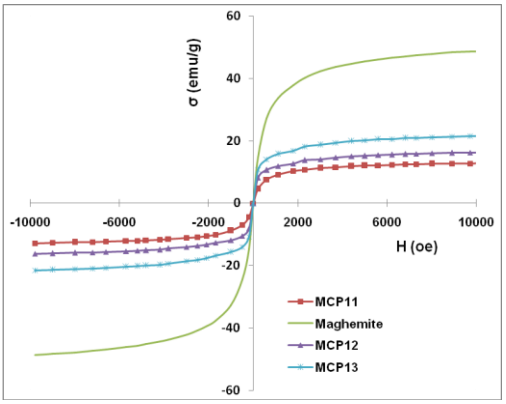

Fig. 8. The magnetization curves of the microparticles (samples MCP) and for magnetic material as recorded by VSM.

Table 3. Values for prothrombin time (PT), quick index (IQ) and international normalized ratio (INR) of magnetic polymer particles.

Sample code	PT (s)	IQ (%)	INR
MCP4	17.5	88.6	1.08
Blood	16.2	54.0	1.68

The quick index was calculated with equation (3)

$$IQ = (PT_C/PT_{nano})\cdot 100 \quad (3)$$

4.8 Particles Toxicity

The toxicity of the magnetic polymer particles was evaluated with the average lethal dose (LD_{50}). The lethal dose (LD_{50}) for magnetic polymer particles, sample MCP4, is 4372 mg/kg body, corresponding to the interval of low toxicity according to **Hodge and Sterner Scale of toxicity.** The values obtained for all particles did not vary much compared with sample MCP4; therefore, the magnetic polymer particle are in the category of low toxic, so they can be used as drug targeting in biomedical applications.

4.9 Swelling experiments

The loading and the release of drugs from magnetic polymer particles are realized by diffusion so they are also influenced by the swelling of the crosslinked microparticles. The swelling degree (presented in Table 2) is influenced by the preparation parameters of magnetic polymer particles and depends by many factors, such as the crosslinking density, the hydrophobycity of the materials, the ionic strength, as well as the composition of particles. For all the formulations, the swelling degree increases directly proportional with the average diameter of the particles.

The content of CS in prepared particles varies significantly only for particles prepared by variation of

PVA/CS ratio and polymers/maghemite ratio. For all the particles, the percent of the chitosan from particles is higher than the PVA content (or the maghemite content) in final particles composition. This fact suggests that the polysaccharide is more reactive than the synthetic polymer during the crosslinking reaction. Furthermore, PVA, through the hydroxyl groups, has practically no reactivity towards ionic crosslinking agent and has less reactivity towards covalent crosslinking than amino groups of the CS [30] and as consequence it is washed during particles purification [26]. The increase of the chitosan content of particles leads to decrease of maghemite content and to increasing of swelling degree. This behavior is more pronounced in the case of particles prepared by varying the parameter PVA / CS ratio (Fig. 9a) or polymers / maghemite ratio (Figure 9b).

Also, for the formulations MCP6, MCP4, MCP7 (prepared by variation of **PVA/CS weight ratio**) is obvious that higher amount of CS in particles induces higher swelling degrees, due to more hydrophilic nature of CS than PVA, which allows the IPN matrix to absorb higher amount of water.

The increase of the **ionic crosslinking amount** in respect to the polymer content in the initial mixture induce the formation of a denser network (that leads to a higher content of CS in the particles) and determine a decrease of swelling degree [26, 31].

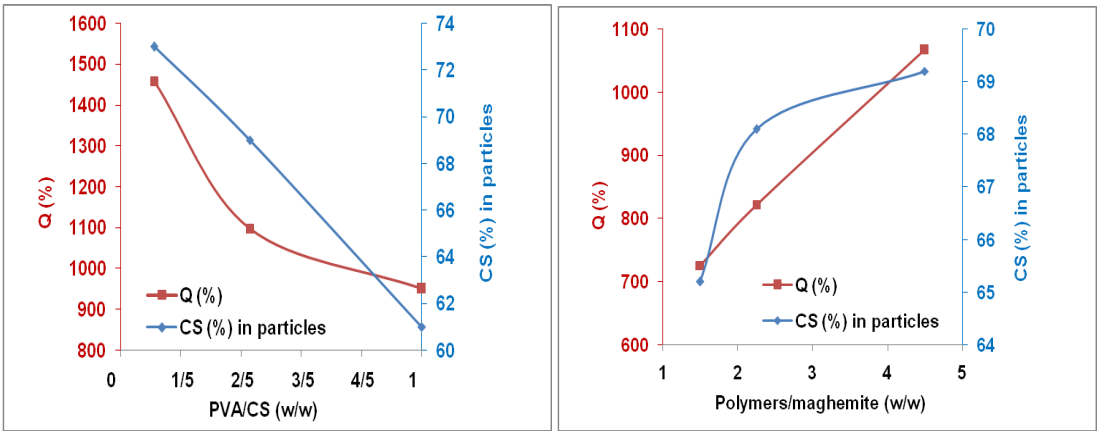

Fig. 9. Correlation between CS content in prepared particles and the swelling degree (Q) of magnetic polymer particles versus preparation parameters: a) PVA/CS (w/w) and b) polymers/maghemite (w/w).

The increase of polymers/maghemite ratio induces an increase in CS content and at the same time, a decrease in maghemite content in prepared particles, due to the repulsions between the protonated amino groups of CS in acid medium and the positively charged maghemite nanoparticles, affecting the inclusion of maghemite in polymer matrix. The particles prepared with bigger amount of maghemite have the lowest swelling degree (Q=726%).

4.10 In vitro drug loading studies

The 5-FU was included in particles by diffusion, so was found a good correlation between drug quantities and the swelling degree of the particles in aqueous media (pH 7.4), as it was already reported for different hydrophilic polymers networks in previous studies [21, 32].

The drug loading capacity of the magnetic polymer particles follows a similar tendency as the swelling degree variation; the drug is retained into the microparticles by a diffusion mechanism, like the water molecules (Table 2). The encapsulation efficiency (%) of drug was between 44 and 76%, depending on particles properties and on amount of drug in diffusion medium.

4.11 Drug release kinetics

In Figure 10 are reported the release kinetics of 5-FU in alkaline environment PBS, pH=7.4) for the sample MCP4 (red line for experimental data and blue line for kinetic equation such as first-order Weibull model). The drug release profile from the particles is constituted of two different steps: an initial rapid phase ("burst effect") for the first 400 minutes of release, followed by a second slower phase for the drug release, for the next 1000 minutes, characterized by a constant release rate. Probably, the release of drugs adsorbed on the particles surface determine the burst phase, while the release of drugs entrapped in the particles IPN network induce the slower step.

The data obtained from *in vitro* release kinetic were fitted to kinetic equation such as first-order Weibull model. As clearly appreciable, it was displayed a drug release profile (line red for experimental data) that follows the Weibull model (line blue in Figure 10). The Weibull equation (4) describes quite well drug release data for all the samples:

$$Q_t/Q_\infty = 1- \exp(-a \cdot t^b) \quad (4)$$

where *a* and *b* are constants, Q_t and Q_∞ are cumulative amounts of drug released at time t and infinite time, respectively [33].

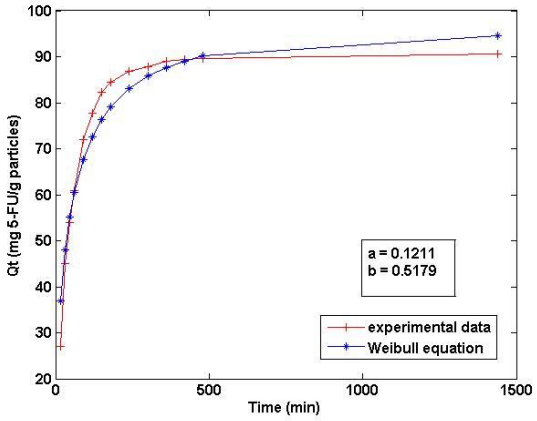

Fig. 10. The 5-FU release kinetic for MCP4 sample. Experimental data is presenting with red line and blue line is the corresponding fitting by Weibull model.

The value of *b* (0.5179) obtained inform as that the release follows Fickian diffusion in fractal space.

For the first 60% released drug, the Korsmeyer-Peppas equation (5) was applied

$$Q_t/Q_\infty = k \cdot t^n \quad (5)$$

where *k* is a kinetic parameter that represents the interactions between drug and polymer, *n* is a parameter who characterize release mechanism and depends on the system geometry. The *n* value was determined from the experimental data and informs on the validity of the Fickian transport mechanism. The value of the release exponent obtained (n=0.59) is close to the limit of 0.5 value, which indicating that the 5-FU release processes is close from Fick model for magnetic polymer particles. The similar behavior was finding by others authors, for other magnetic polymer particulate systems [25, 34].

5. Conclusions

New magnetic polymer microparticles were prepared by double crosslinking in reverse emulsion technique. The magnetic polymer microparticles possess a narrow size distribution from 0.71 to 2.55 μm and exhibiting superparamagnetism with high saturation magnetization and no remanence. The particles final composition and morphology can be modified by changing the preparation parameters. The microparticles can be applied in different fields of medicine and biotechnology.

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