Gelatin-hydroxyethyl cellulose magnetic microparticles as drug carriers: preparation and characterization

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The aim of this study is the preparation and characterization of magnetic polymer microparticles suitable for the treatment of certain cancer diseases. For their preparation the crosslinking method in reverse emulsion has been used, leading to an interpenetrated network based on both natural polymers gelatin and hydroxyethyl cellulose. Crosslinking was carried out with glutaraldehyde within aqueous droplets containing polymers mixture and maghemite, dispersed within toluene in presence of surfactants (Tween 80 and Span 80). The influence of the following preparation parameters on the physicochemical properties of the microparticles was studied: polymers mixture to maghemite weight ratio, gelatin to hydroxyethyl cellulose weight ratio and polymers mixture to crosslinking agent weight ratio. The particles were characterized for their morphology in terms of size distribution (laser diffraction analysis), and shape/surface aspects (scanning electron microscopy), magnetic properties (magnetisation), structure (Fourier transform infrared spectroscopy), chemical composition (Kjeldahl analysis and thermogravimetric analysis), loading and release of methotrexate, as antitumoral drug. The very low toxicity of these magnetic polymer microparticles makes them suitable for being used as drug carriers.

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1. Introduction

Magnetic nano- and microparticles are generally prepared from substances with a very strong magnetic character (iron, iron oxides – magnetite or different ferrites) and they present a strong magnetic moment due to which they can be guided by external magnetic field. Magnetic nano- and microparticles are widely used in biomedical applications [1, 2], such as diagnosis, bioseparation of human blood components [3], purification of biomolecules, blood vessel embolization, as magnetic resonance imaging contrast enhancement agents [4, 5], blood detoxification of the patients suffering from hepatic/renal deficiencies [6, 7], addition of antibody attached to superparamagnetic nanoparticles [8], locoregional radiotherapy [9], electromagnetically induced intratumoral hyperthermia [10], superparamagnetic contrast agents in nuclear magnetic resonance imaging [11], drug magnetic targeting [12].

Polymer magnetic particles are high-tech materials with applications in the field of biomedicine, important studies being dedicated to targeted drug delivery for the treatment of cancer. By association of drug with magnetic particles in a polymeric matrix, this type of system can be administered by a parenteral route (intra-arterial or intravenous) and, due to their magnetic properties, can be manipulated by an external magnetic field to the disease (tumor) area.

A tremendous amount of research is devoted to the covering of the magnetic particles with biocompatible and biodegradable natural or synthetic polymers [12-27]. The polymeric cover layer of the magnetic particles assures the possibility of inclusion or of chemical binding to biologically active substances (drugs).

The classic administration of drug to treat some diseases on oral or intravenous routes is still the most widely used today, but these traditional methods are associated with some disadvantages, especially with the uniform distribution of drug in the body, including in the healthy areas. Partially, these situations can be avoided by using microparticles as carrier drug systems which are easy delivered via magnetic way; the microparticles are able to deliver the drug directly targeting the affected tissue.

The materials (magnetic or polymeric nature) used in microparticles preparation must have a high degree of biocompatibility, while the microparticles must have an adequate morphology (shape, dimensions and dimensional polydispersity) and high capacity of drug loading.

The purpose of this study was to prepare and to evaluate the magnetic polymer microparticles based on hydroxyethyl cellulose, gelatin and maghemite as a new controlled release system for methotrexate, as an antitumoral drug. For improving the biological properties and the drug loading capacity, blending of two polymers is often studied for preparation of particles [27-30].

Another goal of this work was to investigate the dependence of drug inclusion and release on the preparation conditions.

These two polymers have previously been successfully used in the preparation of polymer particles by using the same preparation method (crosslinking in reverse emulsion method) [31], which led us to the idea of preparing magnetic polymer particles. To the best of our

knowledge such magnetic polymer particles have not been described in the literature.

Gelatin is a natural protein prepared by hydrolysis of collagen, a fibrous material that exists in bones, skin and connective tissues of animals [32]. Gelatin is well-known biocompatible and biodegradable polymer, widely used in various pharmaceutical applications for the preparation of tablets, emulsions, surgical sponges, ointments, salves, jellies, suppositories, plasma substitute for medicines, dietary/health supplements, syrups, etc [28, 29]. The macromolecular chains of gelatin present many amino and carboxylic groups, which assure an amphoteric character for this polymer. Also, due to the functional groups, gelatin may easily crosslink with different crosslinking agents.

Hydroxyethyl cellulose is a non-ionic, water-soluble and non-toxic carbohydrate polymer used extensively in pharmaceutical areas [30, 33]. It is available in a wide range of viscosity grades, but has poor solubility in organic solvents. Also, HEC is used as a polymeric surfactant to protect colloid in acetic alkene emulsion polymerization, to improve the stability of polymer in wide pH range, as dispersant in suspensive polymerization [34].

Iron oxide Fe_3O_4 (magnetite) or its oxidized version, γ-Fe_2O_3 (maghemite) are known as the most common magnetic materials used for preparation of biomedical devices. They are generally used as a core covered with a polymer or uniformly dispersed within a matrix containing a natural or a synthetic polymer [35]. Magnetite [36] is a material widely used as a magnetic resonance imaging contrast enhancement agent due to is biocompatibility. Maghemite nanoparticles are used in biomedicine, because they are biocompatible and non-toxic to humans, while their magnetism allows remote manipulation with external fields [37].

Methotrexate is an antimetabolite and antifolate drug and it is used for chemotherapy, in treatment of a number of cancers including: breast, head and neck, leukemia, lymphoma, lung, osteosarcoma, bladder and trophoblastic neoplasms.

2. Experimental

2.1 Materials

Gelatin from porcine skin, type-A (GEL) and hydroxyethyl cellulose (HEC) were provided from Sigma-Aldrich. Glutardialdehyde (25% aqueous solution) for synthesis (AG), acetone, toluene, n-hexane, Span® 80 and Tween® 80 were purchased from Merck, Germany. Methotrexate (MTX) was provided from S.C. Antibiotice S.A. Iasi, Romania. Glacial acetic acid p.a. grade was received from Chemical Company, Romania. All solutions were prepared with 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

Maghemite based cationic ferrofluid was obtained in our laboratory according to Massart method [38]. The magnetite nanoparticles were prepared 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 presented the pH environ 3, the maghemite concentration was 9.56 % (w) and the average particle diameter was 43.4 nm.

2.2.2. Preparation of gelatin-HEC magnetic particles

Magnetic gelatin-hydroxyethyl cellulose microparticles were prepared by w/o emulsion crosslinking method using glutaraldehyde as crosslinking agent.

Briefly, the procedure for microparticles preparation is further presented. 2% wt. of polymer solution was prepared by dissolving HEC in double distilled water by continuously stirring at 80°C until the complete homogenization and then gelatin was added in the above solution cooled down to room temperature and stirred overnight at 40°C. This solution was acidified with glacial acetic acid to the pH of the ferrofluid (pH = 3); 2% Tween 80 was added as stabilizing agent. In a glass reactor equipped with a mechanical stirrer, the organic phase (the ratio aqueous/organic phase was 1/4) in which the hydrophobic tensioactive Span 80 (2% w/w) dissolved in toluene was added. Under vigorous stirring, the solution of maghemite (ferrofluid) was added, followed by the addition in fine drops of the aqueous phase (pH = 3) composed of HEC, GEL and hydrophilic tensioactive agent, Tween 80, was performed. The obtained w/o emulsion was maintain under stirring for another 30 minutes, then the solution of glutaraldehyde was added dropwise, the crosslinking reaction was allowed to take place for 4 hours at room temperature and 550 rpm stirring speed. When the crosslinking 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 the excess of the crosslinking agent. The microparticles were finally dried from n-hexane at room temperature. The following preparation parameters were studied from the point of view of their physico-chemical characteristics: GEL–HEC w/w ratio (in 2/3, 1/1, 3/2 and 1/0 w/w ratio), polymers-maghemite w/w ratio (3.25/1, 2.5/1, 2/1 and 1/1) and polymers-crosslinker w/w ratio (6/1, 10/1, 14/1 and 20/1).

3. Characterization

3.1 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used for structural characterization of the magnetic polymer microparticles. The FTIR spectra were recorded for HEC, GEL and magnetic polymer particles, using a Digilab Scimitar FTS 2000 FTIR spectrophotometer, to confirm the formation of interpenetrated structure and to demonstrate the presence of the maghemite into the prepared particles. FTIR spectra of plain HEC, plain GEL, plain maghemite and magnetic polymer particles based on gelatin (GEL) and hydroxyethyl cellulose (HEC) 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 500 cm^{-1}.

3.2 Composition of magnetic polymer particles (gelatin and maghemite content)

The gelatin content of the magnetic polymer microparticles was analyzed by the nitrogen content determinations by using Kjeldahl method. First it was determined the nitrogen content of the gelatin and the nitrogen content of the prepared particles and then, these values were correlated in order to find the gelatin content of magnetic polymer particles.

The maghemite content of the magnetic polymer microparticles was determined by thermogravimetric analisys, using a Mettler Toledo derivatrograph, characterized by a 20 ml/min flow, with a thermobalance between 25 and 900°C, at a temperature rate of 10°C/min, under nitrogen atmosphere. The weight of the samples was situated between 3 – 5 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 STAR software developed by Mettler Toledo. The thermal tests were accomplished for gelatin, HEC and for magnetic polymer microparticles, to calculate the γ-Fe_2O_3 content.

3.3 Magnetic properties

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

3.4 Scanning electron microscopy study

Scanning electron microscopy (SEM) was used to determine the size, shape and surface morphology of the magnetic polymer microparticles. SEM images were taken using a TM 3000 HITACHI scanning electron microscope, at different magnification (8.000-25.000 times).

3.5 Particle size measurements

The size distribution of magnetic polymers microparticles was determined by laser diffraction analysis using a Shimadzu SALD-7001 particle size analyzer. The magnetic polymer particles were suspended in hexane and dispersed in an ultrasonic bath for 3 minutes before sampling, in the following conditions: particles concentration 0.01 mg/ml, at room temperature, under constant stirring.

3.6 Particles toxicity

All substances intended for use in animals and humans require the evaluation of toxic side effects. This is a "must know" property also for magnetic polymer particles. The toxicity of the magnetic polymer microparticles was evaluated by measuring the lethal dose (LD_{50}). The LD_{50} is one way to measure the short-term toxic potential (acute toxicity) of materials. LD_{50} is the amount of a material, given all at once, which causes the death of 50% of a group of test animals. The LD_{50} value is expressed as the weight (mg) of the material administered per kilogram body weight of the animal and it states the test animal used and route of exposure or administration. The magnetic polymer microparticles were suspended in Tween 80 and administered via the intraperitoneal way on rats weighing 20 ± 2 g, according to the classical laboratory methodology. The value obtained for LD_{50} of the microparticles was interpreted according to Hodge-Sterner toxicity scale [39].

Table 1. Hodge and Sterner toxicity scale.

Description	LD_{50} (mg/kg)
extremely toxic	<1
highly toxic	1-50
moderately toxic	50-500
slightly toxic	500-5000
practically non-toxic	5000-15000
relatively harmless	>15000

3.7 *In vitro* degradability tests

Taking into account the gelatin presence in the microparticles which is a collagen derivative, the degradation of magnetic polymer particles was evaluated by measuring the amount of α-NH_2 groups (mmol/ml$\times 10^{-10}$) after enzyme action, using the ninhydrin test [40]. The enzyme responsible for degradation of native helical collagen fibrils *in vivo* is collagenase. The degradability of particles in presence of the enzymes was studied using collagenase from *Clostridium histolyticum* (EC 3.4.24.3, US Biological, USA). The collagenase activity is 125 units/mg dry weights.

The preparation of ninhydrin reactive was realized by dissolving $SnCl_2 \cdot 2H_2O$ (0.032 M) in citrate buffer solution pH 5.0, and by dissolving the ninhydrin in 2-methoxyethanol (4%). These solutions were mixed and kept under nitrogen atmosphere for 15 min. The magnetic polymer particles samples were incubated in PBS (0.01 M, pH=7.4), containing 0.01% collagenase and maintained in a shaking water bath at 37°C. From time to time, after centrifugation, 1 ml of sample solution was withdrawn (and replaced with 1 ml of collagenase solution), mixed with ninhydrin reactive (5 ml) and kept in boiling water (100°C) for 20 min. Then, the samples were introduced in ice bath for cooling to 25°C and after that the samples were mixing with water/2-propanol solution (1:1, v:v). The optical absorbance was recorded (570 nm, UV–VIS spectrophotometer Boeco, Germany). For calculating the degradation degree, the calibration curve of gelatine and the equation (1) were used:

$$DD = (N_p/N_G)\cdot 100\% \quad (1)$$

DD is the degradation degree and N_p, N_G are the concentration of the α-NH_2 groups (mmol/ml$\times 10^{-10}$) for polymer magnetic particles, respectively for pure gelatin.

3.8 Hemocompatibility tests

For evaluation of hemocompatibility, the prothrombin time (PT) was measured and the international normalized ratio (INR) of blood in contact with magnetic polymer particles was calculated. For this purpose, an amount of integral blood was collected by venous puncture from a non-smoker, healthy volunteer. This blood was incubated with anticoagulant (aqueous sodium citrate 3.8 % w/v; ratio 1/9 v/v). A suspension of magnetic polymer particles in physiological serum (0.5 ml; 0.02 % w/v) was added to 3 ml blood to achieve a concentration of 0.015 mg/ml particle in blood). The preparation of control samples was realized by adding 0.5 ml of physiological serum to the same volume of integral blood. The samples and the controls were incubated at 37°C, under gentle shaking, for 30 min. After that, the particles were separated from blood cells by centrifugation at 1000 rpm, for 10 min. PT in blood plasma was determined as mean of three values, using an blood tester (ACL 100) and a PT-Fibrinogen kit (Biodevice, Italy) with an International Sensitivity Index (ISI) = 1.06.

The INR parameter was calculated as ratio between the particles prothrombin times (PT_P) and the control samples prothrombin times (PT_C) raised to the power of the ISI value:

$$INR = (PT_P / PT_C)^{ISI} \quad (2)$$

3.9 Swelling experiments

The gravimetric method for determination of the maximum swelling degree of the particles was used (preliminary tests showed that this is the appropriate time interval). The swelling behavior was studied by measuring the weight of particles immersed in phosphate buffer solution, PBS (pH=7.4). To ensure the complete swelling, the samples, weighted (w_0) in Eppendorf tubes, were allowed to swell for 24 hours. The swollen samples were collected by ultracentrifugation and excess of liquid was removed by carefully blotting with filter paper. The microparticles (dry and swollen) were weighed with an accuracy of ± 0.0001 g on an electronic microbalance.
The swelling degree was calculated using the following equation:

$$Q_w(wt\ \%)=[(w-w_0)/w_0]\cdot 100 \quad (3)$$

where w and w_0 are the weight of the swollen and of the dry magnetic polymer microparticles, respectively.

3.10 In vitro drug loading studies

The swollen magnetic polymer microparticles in phosphate buffer solution (pH =7.4) were suspended in a solution of methotrexate prepared in PBS, with 5 mg/ml concentration, at room temperature, for 24 hours. Particles already swollen have been used for these experiments in order to avoid the competition between water and drug molecules. After drug loading, the particles were separated from supernatant by ultracentrifugation. The methotrexate amount present in the medium was determined using a Spectrophotometer NanoDrop ND-1000. A calibration curve of methotrexate at 302 nm wavelength was previously realized. 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.

3.11 Drug release kinetics

Drug release was investigated by dialysis, by measuring the drug concentration in the supernatant after the immersion of the precisely weighed loaded microparticles for 3000 minutes time interval. 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 methotrexate 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 302 nm.

4. Results and discussion

Both polymers used for this experiment present many functional groups able to react with the crosslinking agent. Gelatin presents amino groups which react with carbonil groups of the AG forming imine type bonds. HEC presents hydroxyl groups which form with the carbonil groups of

the AG either semi-acetal or acetal bonds. The obtained polymer microparticles will have a complex structure, of an interpenetrated-interconnected network (IPN). The possible reactions are displayed in Fig. 1.

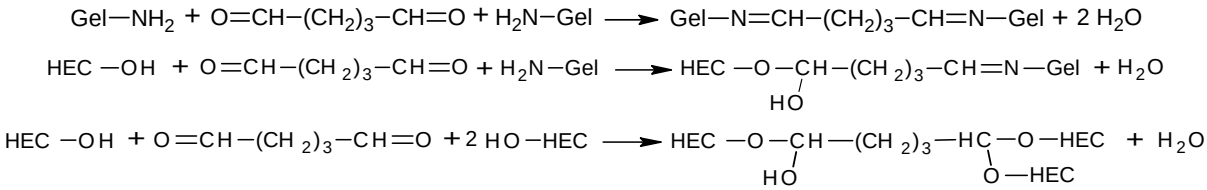

Fig. 1. Schematic representation of crosslinking reactions of gelatin and HEC with glutaraldehyde.

4.1 Fourier transform infrared spectral study

FTIR spectral data show the specific absorption bands of two natural polymers, proving that the covalent crosslinking has been realized and confirm the presence of the maghemite in microparticles. FTIR spectra of GEL, HEC and magnetic polymer particles are compared in figure 2. In case of gelatin, a characteristic band due to N-H stretching is observed at 3423 cm^{-1}, N-H bending vibration is a band observed at 1537 cm^{-1}, aliphatic C-H stretching vibrations is observed at 2958 cm^{-1}, aliphatic C-H bending vibrations is observed at 1450 cm^{-1}, the band appearing at 1647 cm^{-1} indicates amide I and bands at 1334 cm^{-1} and 1238 cm^{-1} indicate the C-N bond stretching vibrations.

In case of HEC, a band at 3433 cm^{-1} due to O-H stretching vibrations, a band at 2926 cm^{-1} due to C-H aliphatic stretching vibrations, two bands at 1072 cm^{-1} and 1020 cm^{-1} due to C-O-C stretching vibrations are observed.

In the case of magnetic polymer microparticles, all the peaks appeared both in gelatin and HEC were observed. In addition, a new band was observed at 1523 cm^{-1}, indicating the C=N stretching vibration of the imine group of Schiff base. This band confirms the formation of crosslinking between amino groups of the gelatin and carbonyl groups of the glutaraldehyde. The band at 1029 cm^{-1} is due to the presence of an acetal group resulted from the reaction of AG with hydroxyl groups of HEC. Also, in the FTIR spectra of the magnetic polymeric microparticles, there is a band at 636 cm^{-1} which confirms the presence of the maghemite (γ-Fe_2O_3) in the composition of the microparticles. Thus, FTIR data confirm the successful crosslinking of both GEL and HEC to form IPN network structure in the presence of AG.

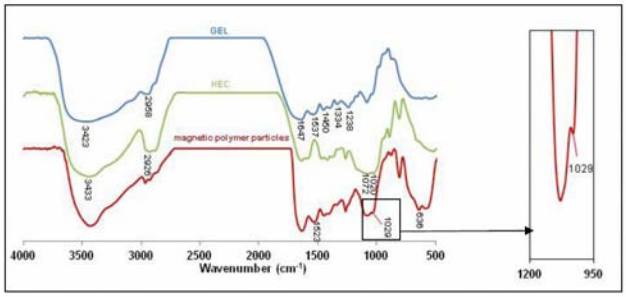

Fig. 2. FTIR spectra for GEL, HEC polymers and for magnetic polymer microparticles, sample GHM_2.

4.2 Morphological study

The shape of magnetic polymer microparticles was studied by SEM. The formulated microparticles present a well defined spherical shape and few aggregates. Their surface is smooth and the particle size is under 3.4 μm (Fig. 3) confirmed by laser diffractometry measurement.

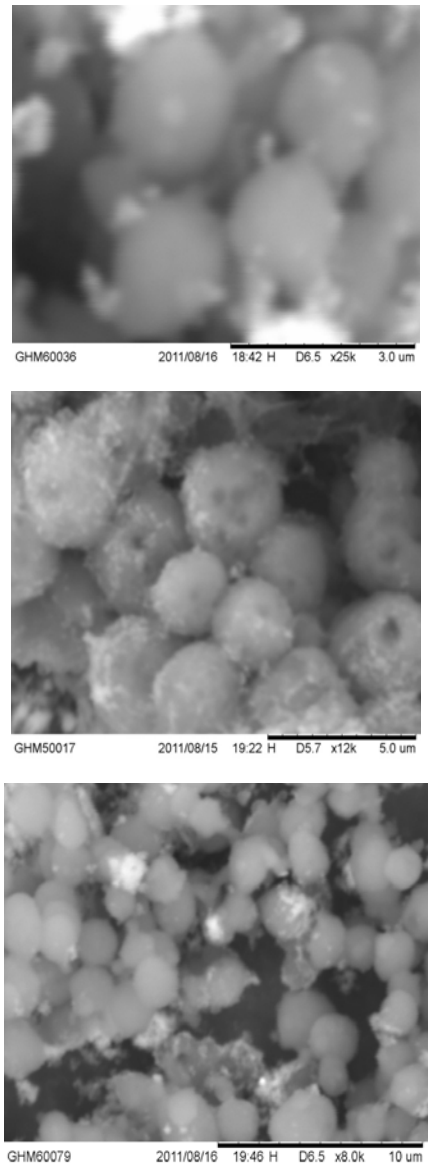

Fig 3. SEM microphotographs of the magnetic polymer microparticles, samples GHM_5 and GHM_6.

4.3 Size of magnetic polymer particles

Average diameter of the maghemite particles is 43.4 nm, and the average diameters of the magnetic polymer particles are presented in Table 2. The mean diameter of the magnetic particles has values situated between 1.57 µm and 3.32 µm, depending on the crosslinking reaction parameters. The interrelationship between the particle size, the composition and the swelling degree on one side, and, on the other side the analyzed reaction parameters will be discussed later in the following section of the paper.

4.4 Composition of magnetic polymer particles

Particles composition (gelatin, HEC and maghemite) is strongly influenced by the crosslinking reaction parameters and by the initial composition of the polymers-maghemite mixture. The first studied parameter was ratio between mixture of polymers and maghemite, keeping constant the ratio between GEL and HEC (1/1).

Table 2. Experimental parameters, swelling degree and average diameter of the magnetic polymer microparticles.

Sample cod	GEL (%)	HEC (%)	polymers/maghemite (g/g)	polymers/AG (g/g)	average diameter (µm)	Q(%)
GHM_2	50	50	3.25/1	10/1	2.09	514.62
GHM_1			2.5/1		1.87	508.53
GHM_3			2/1		1.73	349.12
GHM			1/1		1.57	313.57
GHM_4	40	60	3.25/1	10/1	1.94	348.25
GHM_2	50	50			2.09	514.62
GHM_5	60	40			2.88	569.52
GHM_6	100	0			3.32	580.38
GHM_7	50	50	3.25/1	6/1	1.88	494.08
GHM_2				10/1	2.09	514.62
GHM_8				14/1	2.4	520.74
GHM_9				20/1	2.73	525.15

The **increase of polymers mixture/maghemite ratio** (which leads to an increase of gelatin content in initial mixture) determines an increase of the gelatin content and a decrease of the maghemite content in the final composition of microparticles (Table 3).

Table 3. Composition of the obtained magnetic polymer microparticles.

Sample cod	Composition of particles (%)		
	GEL	γ-Fe_2O_3	HEC
GHM_2	58.69	18.73	22.58
GHM_1	47.09	28.38	24.53
GHM_3	39.45	34.41	26.14
GHM	33.2	38.08	28.72
GHM_4	50.16	17.34	32.5
GHM_2	58.69	18.73	22.58
GHM_5	60.72	31.49	7.79
GHM_6	63.07	36.93	0
GHM_7	62.13	17.05	20.82
GHM_2	58.69	18.73	22.58
GHM_8	51.71	20.07	28.22
GHM_9	47.1	23.91	28.99

The decrease of the maghemite content with increasing the GEL content in particles may be explained by the fact that the cationic character of the protein in acid medium induces repulsion forces with positively charged maghemite nanoparticles, affecting their inclusion. A similar behavior was reported by Tataru et al. [27] for magnetic polymer particles based on gelatin and another polysaccharide, carboxymethyl cellulose.

The swelling degree of magnetic polymer microparticles is higher with the polymers mixture/maghemite ratio from 313% to 514%, due to the increase of gelatin content in the magnetic polymer microparticles. The particle diameter is also higher with the polymers mixture/maghemite ratio and it varies slowly between the 1.57 to 2.09 µm (see Fig. 4).

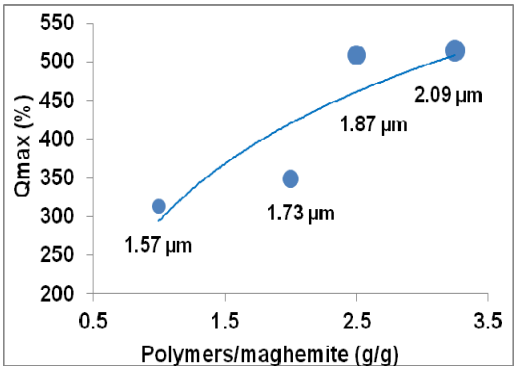

Fig. 4. Correlation between polymers mixture/maghemite ratio, swelling degree and average diameter of the magnetic polymer particles.

Enhancing the **initial content of the gelatin** in the initial polymers mixture leads to an increase of the gelatin content in the final composition of magnetic polymer microparticles, but never exceeded 63.07% (for the

samples GHM_6 prepared with ratio between polymers GEL/HEC=1/0).

Another interesting observation was that GEL content in the particles is always higher than the HEC content, whatever is the ratio between the two polymers (Table 3). This fact demonstrates that gelatin is more reactive than HEC in the crosslinking reaction, the effect is due to the higher nucleophilic effect of amino groups in respect with the HEC hydroxyl groups [41]. On the other hand, as it was already mentioned, HEC is probably involved as a surface stabilizer.

The increase of gelatin content in magnetic polymer microparticles can be correlated with the increase of the swelling degree of microparticles situated between 348% and 580%. Particle size slightly increases with gelatin in the initial mixture (Fig. 5). This could be due to the fact that at higher amounts of gelatin, the viscosity of polymer solution increased, thus producing a little bit bigger droplets during emulsification that lead to bigger particles. The mean diameter of these particles is situated between 1.94 μm and 3.32 μm, and presenting a good polydispersity. These curves present a monomodal distribution of the diameter values, see Fig. 6.

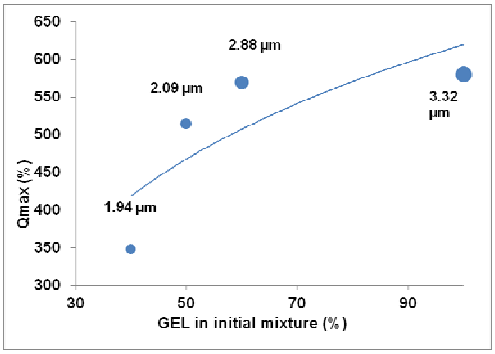

Fig. 5. Correlation between GEL/HEC ratio, swelling degree and average diameter of the magnetic polymer particles.

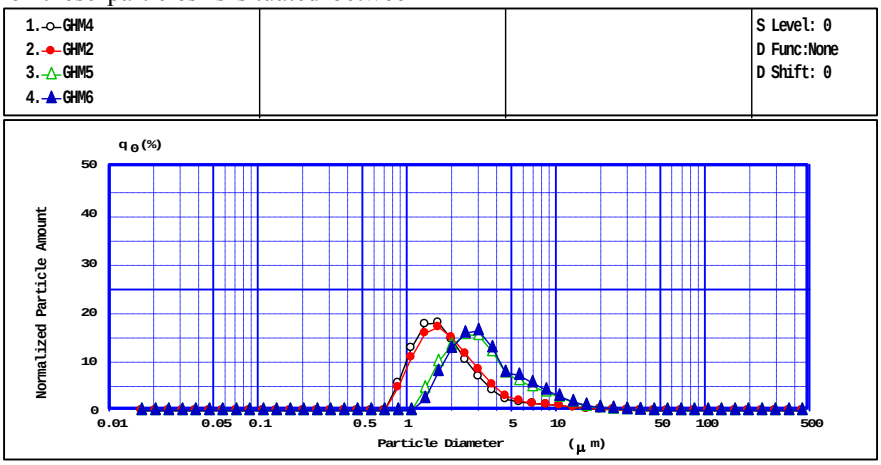

Fig. 6. Typical dimensional polydispersity curves for the samples GHM_4, GHM_2, GHM_5, GHM_6.

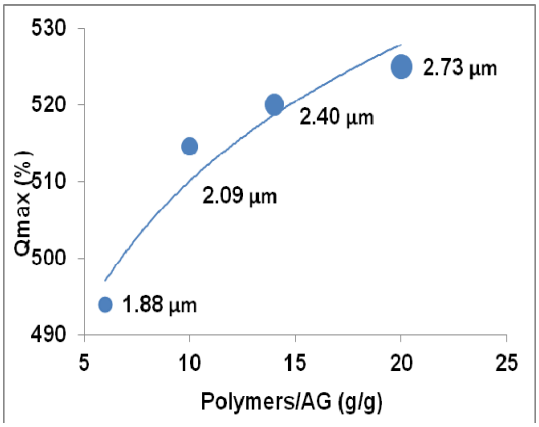

Fig. 7. Correlation between polymers/AG ratio, swelling degree and average diameter of the magnetic polymer particles.

An increased amount of the **crosslinking agent** generally leads to an increase of the network crosslinking density. This determines, on the one hand, the immobilization of more gelatin chains in IPN, so the increase of the gelatin content in the microparticles and, on the other hand, the decrease of the particle size and of the swelling degree. Gelatin content increases from 47% to 62% with the decrease of polymers/AG ratio, and maghemite content is decreasing from 24% to 17% in magnetic polymer microparticles. The average particle diameter is increasing slightly with ratio polymers/AG and it varies between 1.88 μm and 2.73 μm (Figure 7) and the swelling degree is increasing with the same parameter, varying between 494 % and 525 %.

4.5 Magnetic properties

Fig. 8 gives the hysteresis loop and saturation magnetization for two samples of microparticles GHM_2 and GHM_3. The magnetization curve passes through the origin of coordonate system; the absence of hysteresis (M_r=0 and H_c=0), when magnetic field applied, showed that the magnetic polymer microparticles present superparamagnetic properties. It means that they are attracted to a magnetic field and that they retain no residual

magnetism if the magnetic field is removed. The superparamagnetic property of polymer magnetic microparticles is critical for their application in biomedical and bioengineering fields, which prevents microparticles from aggregation and enables them to redisperse rapidly when the magnetic field is removed [42]. It can be seen that the two types of particles exhibit similar overall magnetic behavior, characteristic of soft magnetic materials (particles), with a narrow hysteresis cycle [43].

The values of magnetization of saturation (Ms), for magnetic polymer particles sample GHM_2 are 26.12 emu/g and for sample GHM_3 is 33.24 emu/g.

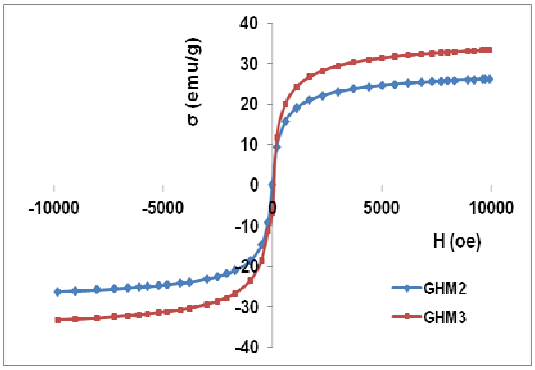

Fig. 8. Magnetization curves of magnetic polymeric microparticles GHM_2 and GHM_3.

The variation of the magnetic measurements results is similar to that measured by thermogravimetric analysis. For example, the sample GHM_2 show a saturation magnetization (M_s) of 26.12 emu/g and the content of maghemite is 18.73%, while the sample GHM_3 present a saturation magnetization (M_s) of 33.24 emu/g and the content of maghemite is 34.41%. Polymers is not a magnetic material, consequently the increase of the saturation magnetization can be considered an indication of the polymers participation in particles composition which contributes as a non-magnetic material to the total sample mass. Nevertheless, all the particles present sufficient magnetization (about 30 emu/g) which makes them suitable for magnetic drug carrier.

The formulated magnetic polymer microparticles have a free flowing nature. When the suspension in acetone of microparticles (image 1) was placed near a magnetic stirring bar (image 2) their magnetic response was very good and they were very fast attracted to the magnet, as can see in figure 9. A color change from brown to transparent was observed when an external magnetic field is applied; the same behavior was observed by Khan [44]. The photos indicate that particles present magnetism and can be collected by the external magnet.

Fig. 9. The magnetic polymer particles in absence of magnetic field (first image) and in presence of magnetic field generated by a laboratory magnet (second image).

4.6 Evaluation of toxicity

HEC and GEL are well known biocompatible and non-toxic polymers. For the preparation of the magnetic polymer microparticles it is necessary to use some toxic chemicals, such as the organic solvent (toluene), the crosslinking agent (glutaraldehyde) and the surfactants (Tween 80 and Span 80). Use of these particles for the medical purpose inside the human body requires the evaluation of their toxicity. The lethal dose (LD_{50}) determined for magnetic polymer microparticles are the values between 4700 and 5000 mg/kg body. LD_{50} values show very low toxicity of the prepared magnetic polymer particles, evaluated from Hodge and Sterner Scale of toxicity (see section particles toxicity).

4.7 In vitro degradability tests

The biomaterials with applications in biomedical field need to be tested in terms as biodegradability in evaluation of controlled drug delivery systems. The aim of degradability tests is to simulate the physiological human body conditions (pH, temperature and enzyme). The rate of drug release from magnetic polymer particles immobilizing the active molecules is also dependent on the rate of degradation of the particles [45]. In the present study, the enzymatic degradation of magnetic polymer particles and the enzymatic degradation of gelatin were studied and the results showed both the material degraded when treated with collagenase, an enzyme. The enzymatic degradation of gelatin, as well as of the prepared magnetic polymer particles was examined over a period of 72 h. The dynamic degradation results of gelatin and of magnetic polymer particles samples (GHM_2) are presented in Fig. 10. The pure gelatin is quickly degraded, while for the magnetic

polymer particles the degradation process is delayed. The complete degradation of pure gelatin was observed in approximately 48 h, at 37°C, in PBS media (pH=7.4) containing collagenase. The prepared magnetic polymer particles are not fully degraded due to their crosslinked structure and due to the presence of HEC and of the magnetic material (about 40% of HEC and maghemite for tested sample). In 48 h, the reduction in degradation degree in case of magnetic polymer particles is 33%, and after 8 days, the enzymatic degradation of sample GHM_2 is 53.48%.

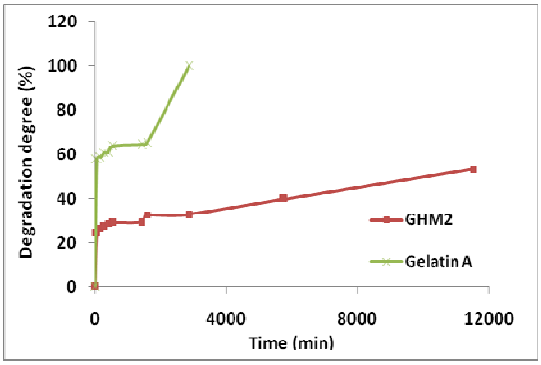

Fig. 10. Degradation degree for gelatin and sample GHM_2 (magnetic polymer particles based on gelatin and HEC).

4.8 Hemocompatibility tests

Considering that the particles are designated to be used inside the human body, as carriers for drug delivery, so they will be in contact with the blood, blood clotting had to be investigated. The interaction of magnetic polymer particles with blood components is evidenced by a change of plasma coagulation properties on incubation with these particles [46]. For evaluation of particles effect on blood coagulation, a standard clinical coagulation assay was used. In order to evaluate 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 result was expressed as the INR [47].

The INR values and coagulation times obtained (Table 4) for magnetic polymer particles (sample GHM_2) and for blood sample are comparables and very similar, proving that the magnetic polymer particles do not inhibit prothrombin activity. Our previous studies [31] for non magnetic polymer particles showed similar results.

*Table 4. Values for prothrombin time (PT) and international normalized ratio (INR) of particles (*IQ is the quick index, calculated with equation 4).*

Sample code	PT (s)	IQ* (%)	INR
GHM_2	18.2	87.57	1.15
Blood	16.2	54.0	1.68

$$IQ = (PT_C/PT_P)\cdot 100 \quad (4)$$

4.9 In vitro methotrexate loading studies

The quantity of methotrexate included by diffusion is in good agreement with the swelling degree of the particles in aqueous media and with particles composition, as it was already reported by the authors in previous studies for different hydrophilic polymers networks [28, 48]. The drug loading capacity of the magnetic polymer particles is following a similar tendency as the maximum swelling degree variation, because the drug is retained within the particles network by the same diffusion mechanism as the molecules of water.

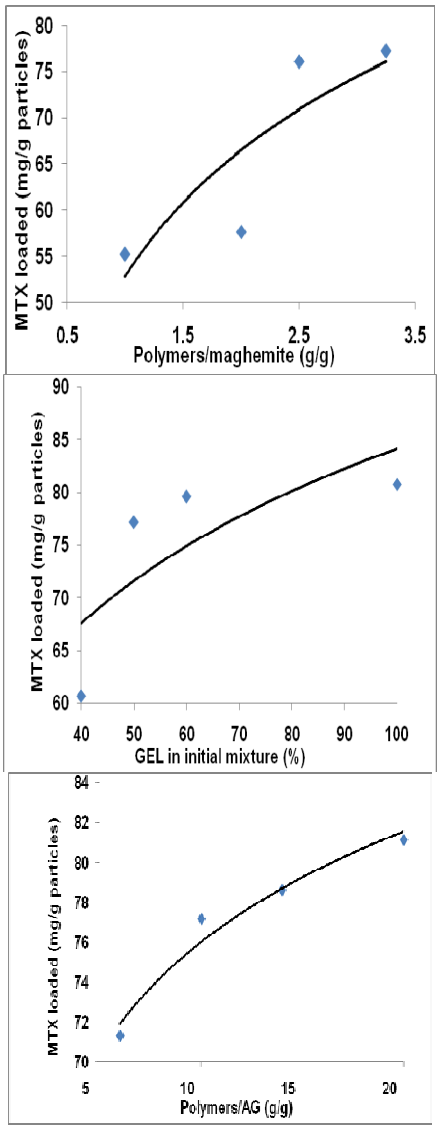

Fig. 11. Influence of preparation parameters on the methotrexate inclusion in magnetic polymer microparticles.

4.10 Methotrexate release kinetics

The release kinetics of methotrexate from magnetic polymer particles GHM_2 is presented in Figure 12. The equilibrium release was achieved after approximately 1000

minutes. The release curve present an initial rapid release followed by a slower release phase. The higher release rate is due to the drug molecules adsorbed on the microparticles surface ("burst effect"), whereas the slower rate of drug release can be attributed to methotrexate entrapped within the microparticles network.

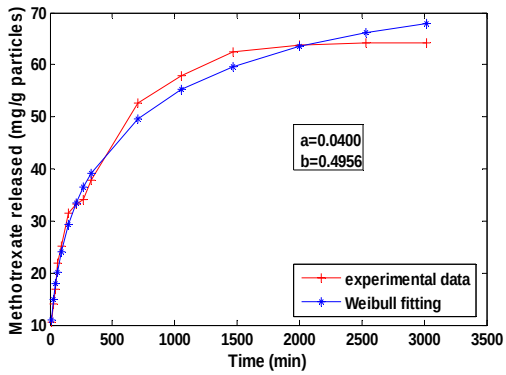

Fig. 12. Methotrexate release kinetic for GHM_2 sample. Experimental data is presenting with red line and blue line is the corresponding fitting by Weibull model.

The methotrexate release behavior was compared with Weibull theoretical model, described by the function:

$$Q_t/Q_\infty = 1 - \exp(-a \cdot t^b) \quad (5)$$

where a and b are constants and Q_t and Q_∞ are cumulative amounts of drug released at time t and infinite time, respectively [49]. The results indicated that the drug release from magnetic polymer microparticles follows the Weibull model.

5. Conclusions

Magnetic gelatin - hydroxyethyl cellulose particles of various sizes were prepared by varying gelatin/HEC ratio (w/w), polymers/maghemite ratio (w/w), polymers/AG ratio (w/w), by polymers crosslinking with glutaraldehyde. The particles were characterized with respect to size, morphology, magnetic properties, chemical composition, toxicity, swelling behavior, loading and release of methotrexate. The obtained polymer magnetic microparticles present superparamagnetic properties and a very low toxicity. The properties of the GEL-HEC magnetic particles are in an interdependence relationship which is very important in modulating these properties for a specific need. Also, the microparticles prepared within the present work proved their biodegradability and hemocompatibility and, together with their methotrexate release profile, make them a perfect candidate for certain applications as magnetic drug carriers.

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