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Dedicated to Professor Alexandru T. Balaban on the occasion of his 80th anniversary

MICROPARTICLES OF HYDROGEL TYPE BASED ON CARBOXYMETHYLCELLULOSE AND GELATIN FOR CONTROLLED RELEASE OF WATER SOLUBLE DRUGS

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The aim of this work is obtaining and characterizing Interpenetrating Network hydrogel based microparticles. They are elaborated by the inverse emulsion co-crosslinking reaction of carboxymethylcellulose and gelatin with epichlorohydrin. The influence of parameters related with the particles elaboration process (ratio of polymers in the mixture, ratio between polymer mixture and crosslinking agent, concentration of polymer solution, duration of crosslinking reaction, stirring intensity…) on their composition, size and swelling ability was studied. Obtained microparticles fulfill the requirements for biomaterials: to be formed from biocompatible polymers, the acute toxicity value (DL_{50}) is high enough to consider these materials as weakly toxic (hence able to be introduced within the organism). They are able to include and release drugs in a controlled way and tests carried out with cefotaxime loaded microparticles demonstrate their antibacterial activity.

INTRODUCTION

Hydrogels represent polymeric networks able to absorb large quantities of water remaining insoluble due to chemical or physical crosslinks between individual polymeric chains.[1,2] They present a series of unique properties which constitute severe **advantages** for their use in biomedical applications: ability to encapsulate biomolecules (including proteins and DNA) due to absence of hydrophobic interaction which may lead to denaturatation of these fragile species;[3] most of them are obtained by reactions which are carried out at room temperature, and the use of organic solvents is rarely necessary; ability to "in situ" gelation simultaneously to the encapsulation ability of active matter; they may be designed to be sensitive to different environment stimuli (pH, temperature[4,5]); they may be designed to be bioadhesive in order to make easier the release of the active matter, mostly by mucus membranes;[6] an increasing circulating time of release system avoiding immune response and decrease of phagocytar activity;[7] they are able to include cells and growth factors.[8]

Hydrogel based particles whose size may vary from tens of nanometers to a few microns can be used to obtain controlled drug release systems thanks to following characteristics:[9] their small size allows to be administered especially by intravenous, intramuscular, subcutaneous injection or using a spray. They present a high contact surface with the external medium leading to a high absorption of the active matter. Moreover, they are very versatile and they may be elaborated in order

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to release the drug with a controlled kinetics. Finally, they have a long storage period compared with other controlled release systems.

This type of microparticles may be prepared either from natural or synthetic polymers. Among natural polymers, polysaccharides are interesting due to the fact they come from living organisms, they are biocompatible, not toxic and present major physico-chemical properties necessary for controlled release applications.[10] From this point of view the most studied polysaccharides are alginate,[11-14] dextran,[15-17]gellan,[18] xanthan[19] and hyaluronic acid.[20, 21] The choice of the material and the synthesis of the polymeric network govern the rate and the release process of the active matter from the hydrogel.[2, 10]

This work presents the elaboration of microparticles based on gelatin (protein obtained by hydrolysis of collagen, GEL) and carboxymethylcellulose (cellulose derivative, CMC), polymers which have attracted considerable attention in biomedical and pharmaceutical domains in the last years.[1, 22-25] Our aim was to obtain microparticles whose water swellability and capacity to include drugs can be modulated; the chosen method to elaborate such hydrogels type microparticles is the covalent crosslinking of polymers in inverse emulsion using epichlorohydrin as crosslinking agent. The work deals with the influence of several crosslinking reaction parameters on GEL composition of such microparticles, on their ability to swell in water and hence, on their capacity to include and release biologically active matter.

RESULTS AND DISCUSSION

The microparticle structure is of an interpenetrating network. As the polymers (GEL and CMC) have functional groups able to react with epichlorohydrin in alkaline medium different crosslinking reactions may occur so that the structure will be complex (Fig. 1). This structure was proven by FTIR and ^{1}H-HRMAS spectroscopies.

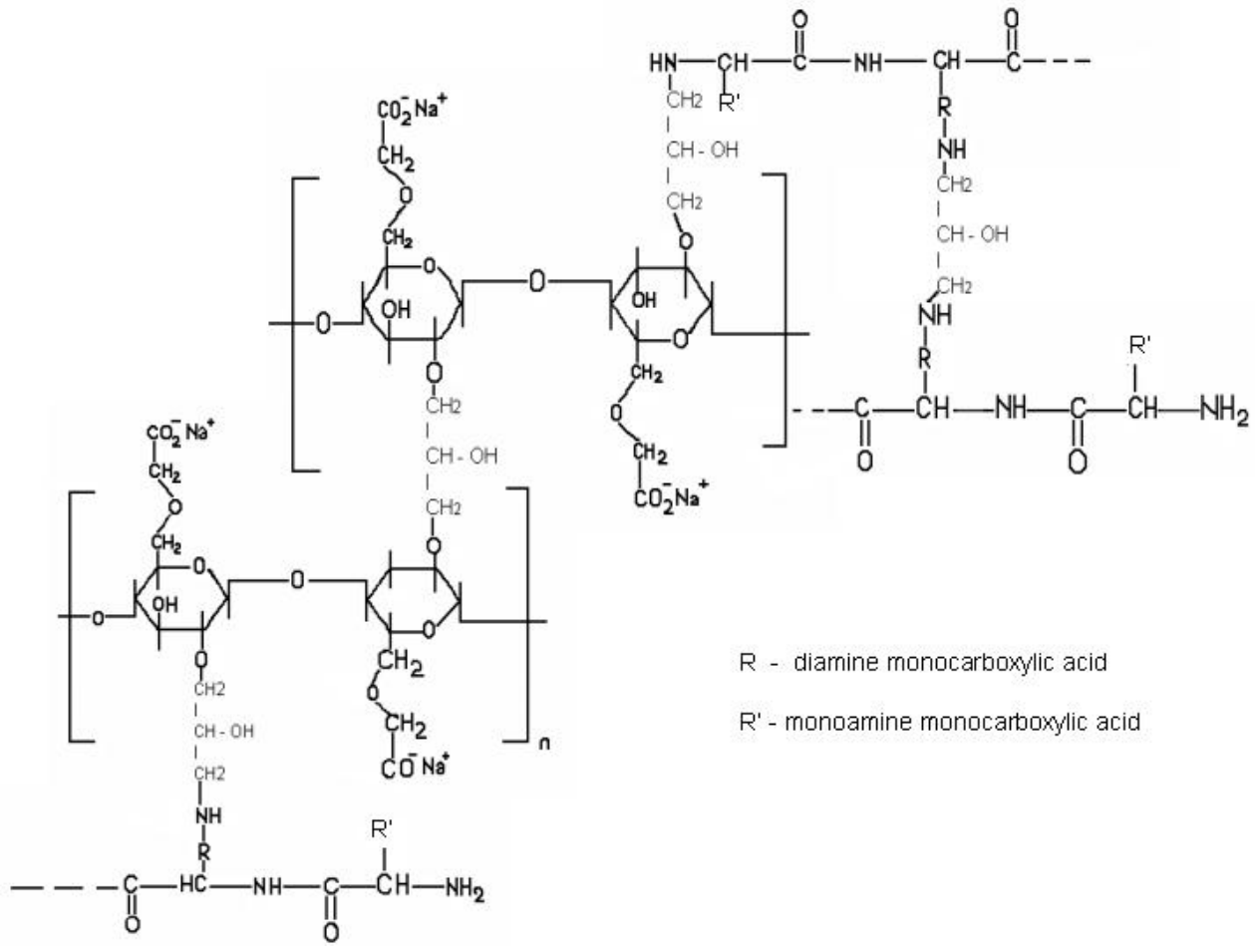

Fig. 1 – Crosslinking reactions of CMC and GEL with épichlorohydrine.

Considering FTIR spectra, the CMC was identified by the bands at 3404 and 3200 cm^{-1} (-OH), 2918 and 2907 cm^{-1} (aliphatic -C-H), 1618 and 1420 cm^{-1} (symmetric and asymmetric extensions of $–COO^-$ group). For gelatin bands appear at 3412 cm^{-1} and 1539 cm^{-1} (-N-H), 2938 cm^{-1}, 1450 and 1402 cm^{-1} (aliphatic C-H). The signal at 1641 cm^{-1} is specific of amide I group when those between 1335 and 1238 cm^{-1} are specific of C-N bond. The new ones appearing between 1113 AND1060 cm^{-1} are assigned to -C-O-C bonds (acetals). [26]

^{1}H-HRMAS spectra confirm the co-crosslinking reactions because the characteristic signals of original polymers (CMC and GEL) are found in the particle spectrum.

The morphology of the particles is rather spherical and the surface is more or less smooth. The particle diameter varies between 6 and 65 μm depending on the studied parameter (Fig. 2).

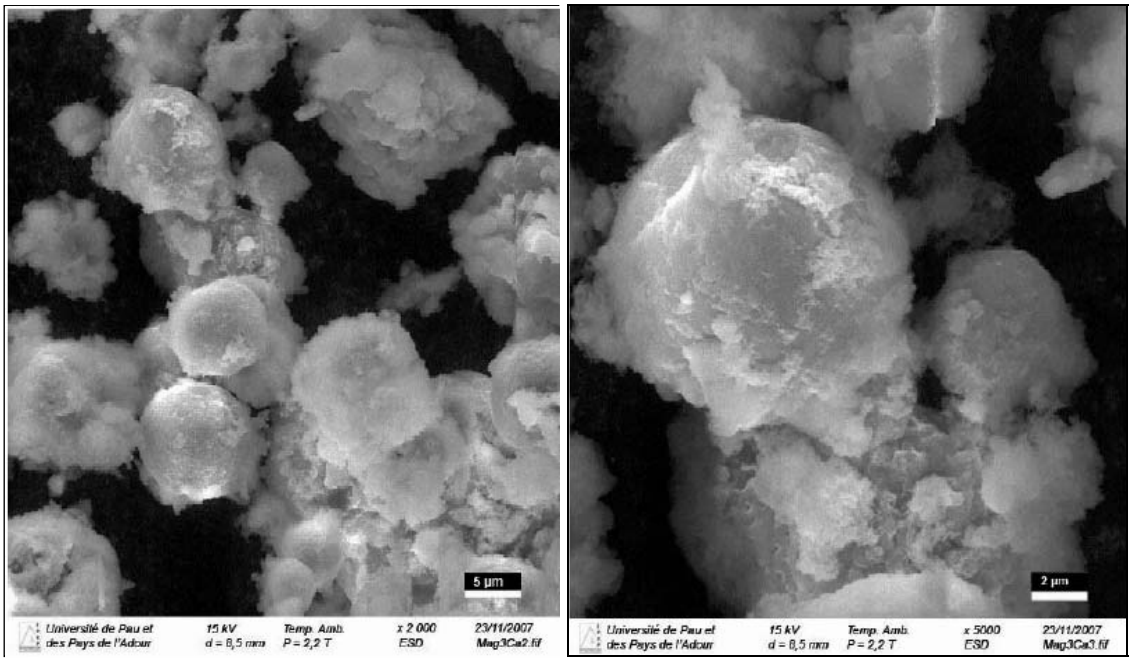

Fig. 2 – Scanning Electron Microscopy images of MEP4 microparticles (resolution 2000X and 5000x).

The optimization of the process to obtain IPN as microparticles assumed the study of the influence of some parameters on their characteristics.

Influence of polymer mixture – EpCl ratio

The analysis performed to determine the microparticle composition has demonstrated that, during the crosslinking of the polymers by EpCl in alkaline medium, it depends on different parameters. As an example, when the ratio between the polymer mixture and the crosslinking agent (r_{PE}) was modified, an increase of the crosslinking agent leads to an increase of GEL in the network. Simultaneously an increase of EpCl leads to an increase of the crosslinking density. As a consequence the structure of the network will be more compact and the size of microparticles will be smaller especially due to the higher participation of the functional groups of CMC (majority component of the particles) compared with GEL. The effect of this parameter on these properties is shown in Fig. 3.

The maximal swelling degree of microparticles was studied in three different media characterized by different pH values, 3.5, 6.2 and 7.4 respectively. Gelatin, due to free $–NH_2$ and –COOH groups is an amphoteric polymer. In aqueous solutions characterized by a pH value larger than the isoelectric point, gelatin owns $-COO^-$ groups and, when pH is decreasing, the amino groups will be protonated ($-NH_3^+$). The carboxymethylcellulose (under sodium salt) is an anionic polymer which possesses carboxylate groups in alkaline media. But when pH values decrease, in acidic media, the free carboxylic and hydroxyl may react by lactonisation and lead to crosslinking.

The evolution of the swelling degree in aqueous solution at different pH values is due to the different behaviors of the polymers forming the network as follows: **(i)** in an acidic swelling medium the free amino groups of GEL are protonated leading to electrostatic repulsions between them, and the network was subject to a relaxation process. On the other hand, the carboxylic groups with the free hydroxyl groups of GEL and CMC lead to a contraction of the network either due to strong hydrogen interactions or by intermolecular lactonisation; **(ii)** In an alkaline swelling medium the carboxylate groups of GEL and CMC provoke a strong expansion of the network due to their high number on the macromolecular chain compared to the previous situation (in acidic medium).

The influence of the composition of particles on the swelling capacity in solutions with different pH values is shown in Fig. 4.

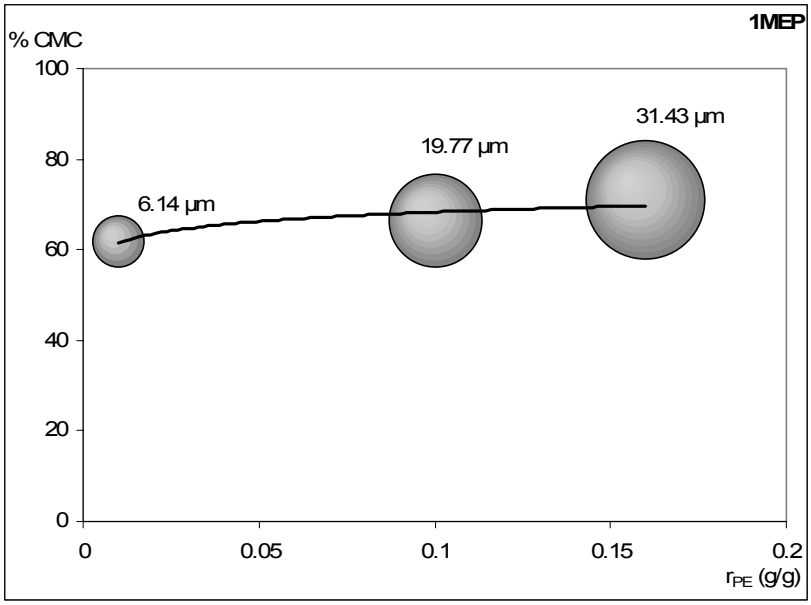

Fig. 3 – Influence of polymer/crosslinking agent ratio on the composition and size of 1MEP (CMC:GEL = 60:40, Cp=2%, w/o=1:4, 700 rpm, t_R=4h).

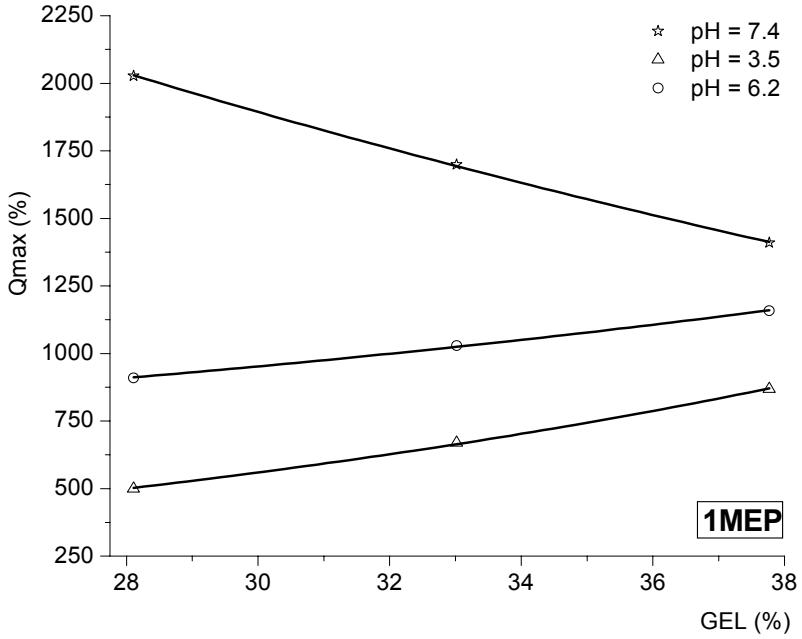

Fig. 4 – Influence of the composition on the maximal swelling degree of 1MEP microparticles (CMC:GEL= 60:40, Cp=2%, w/o=1:4, 700 rpm, t_R=4h).

Following conclusions may be given: **(i)** the maximal swelling ratio is decreasing with the pH of the solution, in agreement with the previous assumptions; **(ii)** for an acidic pH value (3.5 and 6.2) the maximal swelling ratio is increasing slightly as GEL content in the microparticles, behavior which can be explained by the most hydrophilic character of the protein and the protonation of amino groups. At a pH value equal to 3.5 the increase of CMC content in the microparticles leads to a larger decrease of Q_{max} compared to the results within a solution with a pH value of 6.2; **(iii)** in slightly basic solutions (pH = 7.4) the evolution is different with an increase of the swelling ratio due to the increase of the CMC content, related to the intense relaxation of the crosslinked network, determined by the polysaccharide.

Influence of the crosslinking duration

The duration of the crosslinking reaction has no significant influence on the composition of the microparticles. By contrast, the size of the particles is decreasing with the crosslinking time (Fig. 5) due to the increase of the crosslinking density and hence a compaction of the network. After a crosslinking duration of 1 hour the microparticles diameter reaches an average of 44 μm when after 4 hours it is reduced to 17 μm.

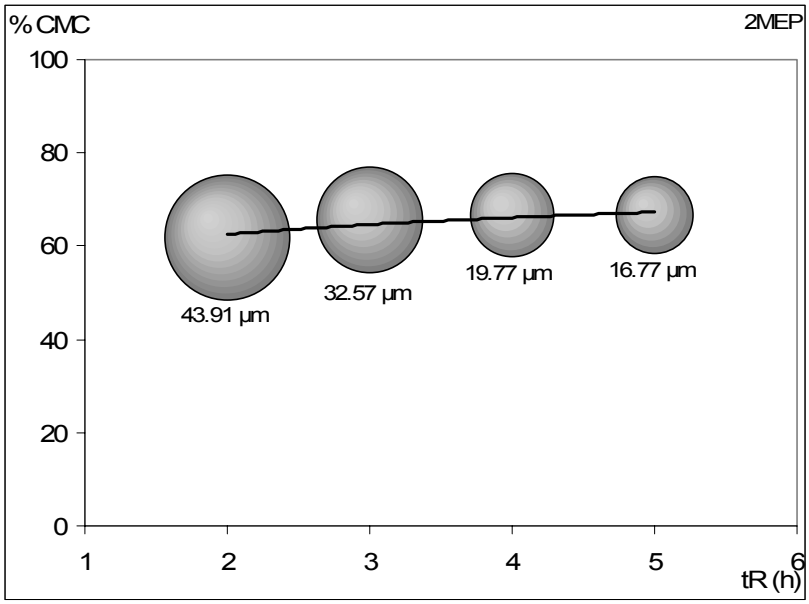

Fig. 5 – Influence of the crosslinking time on the composition and the size of 2MEP microparticles (r_{PE}=0.1, w/o=1:4, CMC:GEL = 60:40, 700 rpm, C_p = 2%).

The swelling ratio is slightly decreasing with the crosslinking time. It is still checked that the swelling ratio is larger in a basic solution than in an acidic one due to the highest content in CMC in the particles.

Influence of the polymer solution concentration

When the reaction parameters are kept constant and the concentration of the polymer mixture in the aqueous phase increases, an increase of the GEL content in the microparticles was observed with this parameter. The higher concentration of the solution leads to a proximity of the polymeric chains, favouring the formation of bridges above all between GEL chains, more and more present in the network (Fig. 6).

The yield in the formation of the microparticles was observed to increase with the concentration of the polymer solution (57% for Cp equal to 1.25% compared to 66% when Cp = 2.5%). Due to the viscosity increase of the aqueous phase, its dispersion in the organic phase is more difficult. As a consequence the resulting emulsion droplets and hence the microparticles will be larger. Analyzing the values plotted in Fig. 6 the dimensions of the particles are very small for low polymer concentrations (1.5%), D_{med}= 6.3 μm, when it is equal to 43.5 μm for C_p=2.25%.

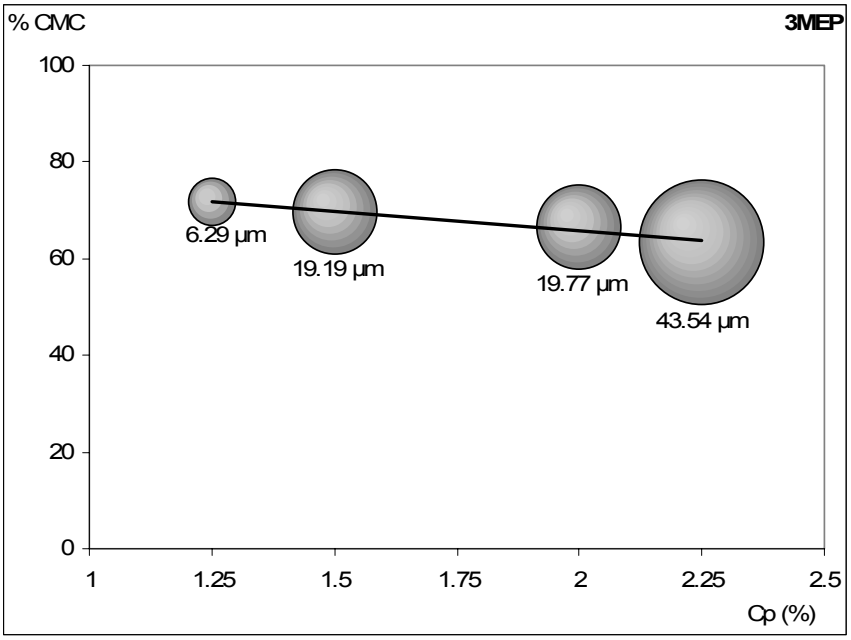

Fig. 6 – Influence of the aqueous phase concentration on the composition and the size of 3MEP microparticles (r_{PE}=0.1, w/o=1:4, CMC:GEL = 60:40, t_R=4h).

The evolution of the maximal swelling ratio as a function of Cp is similar to that of polymer/epichlorohydrin variable parameter (series 1MEP). As a conclusion, in alkaline medium Qmax is decreasing with an increase of GEL content (hence with Cp) when in acidic medium it is increasing with the protein content.

Influence of the hydrodynamic regime

By increasing the rotational speed, *i.e.* an intensive hydrodynamic regime, the size of the particles is decreasing (Fig. 7).

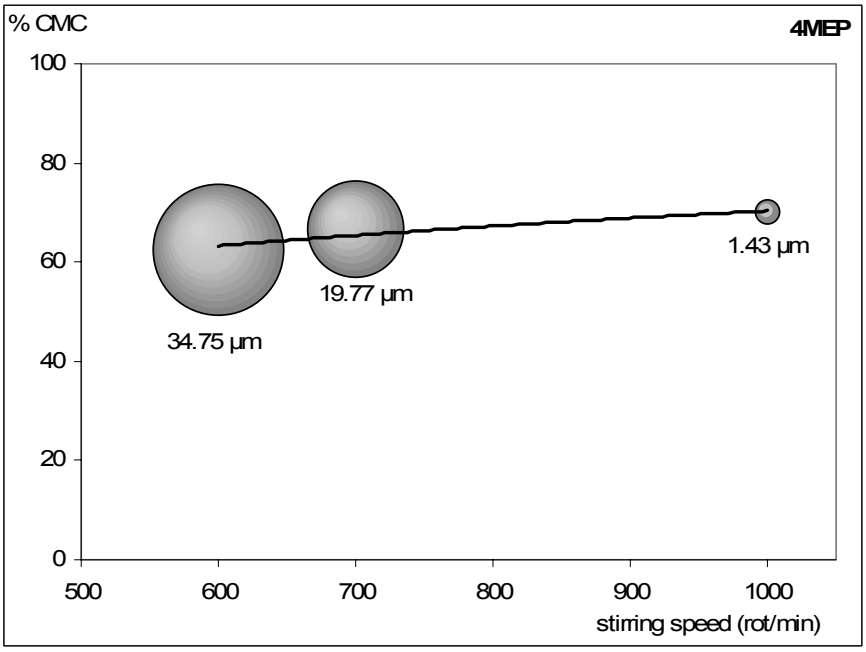

Fig. 7 – Influence of the hydrodynamic regime on the composition and the size of 4MEP microparticles (r_{PE}=0.1, w/o=1:4, CMC:GEL = 60:40, 700 rpm, t_R=2h).

This effect can be explained considering two reasons: **(i)** the dispersion energy created by a stronger stirring ensures the formation of an emulsion containing particles with smaller diameters; **(ii)** the intensification of the crosslinking reactions activated by a stronger stirring leads to an increase of the crosslinking density and compaction of the crosslinked structure, and hence to smaller particles.

The dimension distribution is different of the other series because, in this case, the smallest particles (D_{med}=1.4 μm) obtained with an intense hydrodynamic regime (1000 rot/min) have a normal dimension distribution, monomodal and narrow. This observation shows that the hydrodynamic regime is an important parameter for modeling the obtaining process of microparticles based on CMC and GEL crosslinked with EpCl. The observed diameters vary in a wide range, from 34.8 to 1.4 μm (Fig. 8).

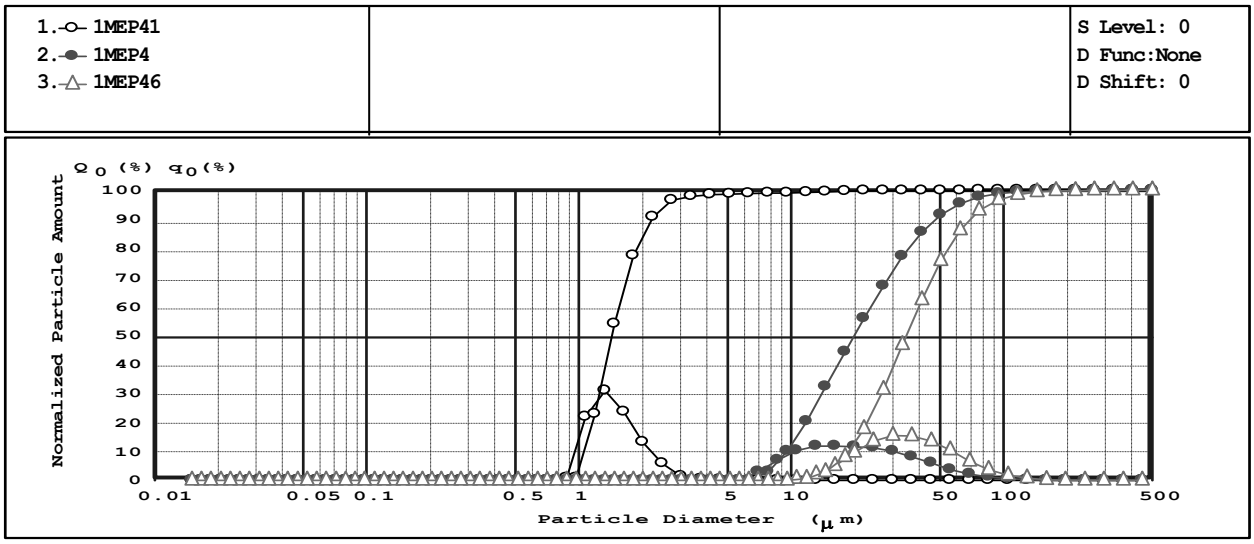

Fig. 8 – Size Distribution of 4MEP microparticles (CMC:GEL = 60:40, Cp=2%, r_{PE}=0.1, w/o=1:4, t_R=4h).

Influence of the ratio between the aqueous and organic phases

The ratio between the phases is a parameter acting mainly on the size of particles. The variation of this parameter was studied keeping the emulsion volume constant (200 mL). Keeping the emulsion volume and the polymer/epichlorohydrin ratio constant assumes an increase of the epichlorohydrin in the organic phase by increasing the aqueous phase volume, the quantity of polymers (C_p= 2%) and decreasing the organic phase volume. Taking into account the difference of epichlorohydrin solubility in the aqueous and organic media, the increase of the epichlorohydrin concentration in organic solution means an increase of the transfer of the crosslinking agent towards the aqueous phase and, finally, an increase of the efficiency of the crosslinking reactions between CMC, GEL and EpCl.

The obtaining yields of particles, calculated for each system (Table 1), in correlation with the increase of the volume of the aqueous phase, which means, as previously mentioned, an increase of the quantity of reacting epichlorohydrin leads to interesting conclusions: **(i)** the crosslinking yield is increasing with the epichlorohydrin quantity (transferred from the organic phase towards the aqueous phase); **(ii)** the crosslinking yield specific to the polysaccharide is larger than the protein one, independently of this situation, being determined by a possible degradation process of GEL (by alkaline hydrolysis of the proteic group) which may be carried out simultaneously with the crosslinking reaction.

If the variation of the microparticles composition is studied (Table 1), it can be noted that for the highest volume of the aqueous phase (w/o=1/2) dispersed in the organic phase, the ratio between the two polymers in the composition of the particles still remains constant. This is due to higher quantities of the crosslinking agent transferred towards the aqueous phase succeeding in crosslinking larger quantities of GEL. As a consequence the obtaining yield of particles is increasing too.

Table 1

Yields of obtaining 5MEP microparticles

Code	W:O (vol/vol)	CMC:GEL Initial (%)	CMC:GEL Final (%)	$\eta_{particles}$, (%)	$\eta_{crosslinking.}$ CMC, (%)	$\eta_{crosslinking}$ GEL, (%)
MEP49	1:5	60:40	76:23	52	60	30.8
MEP4	1:4	60:40	66.5:33	61.5	68.75	50
MEP48	1:3	60:40	61.5:38	69.2	70	65
MEP47	1:2	60:40	60:40	71.6	75	69

The particle diameter is increasing with the volume of the aqueous phase dispersed in the organic phase (Fig. 9). This can be explained by the fact that, when increasing the volume of the aqueous phase, the droplets dispersed in the organic phase are larger, leading finally to more voluminous particles (up to 64.5 μm for w:o = 1:2).

The evolution of the maximal swelling ratio in solutions with different pH values as a function of the content of the most hydrophilic component shows, one more time, its increase with GEL content, which is determined by the ratio between the two phases.

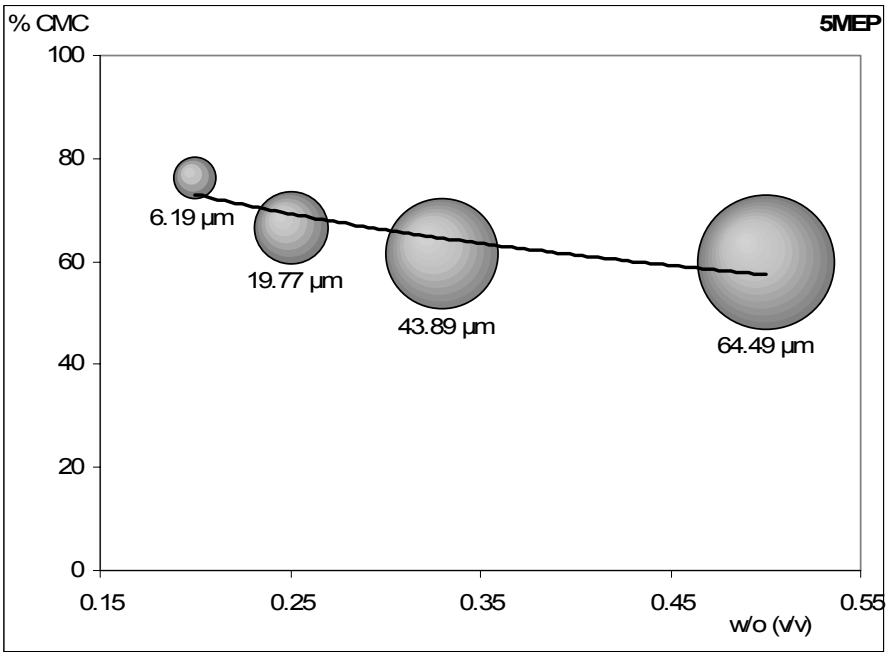

Fig. 9 – Influence of the aqueous phase/organic phase ratio on the composition and the size of 5MEP microparticles (CMC:GEL = 60:40, Cp=2%, r_{PE}=0.1, 700 rpm, t_R=4h).

Biological and biomedical properties of the particles

The acute toxicity of MEP microparticles was evaluated by the determination of the average lethal dose (DL_{50}). Due to the results [(DL_{50}) = 1180 mg/kg body], the microparticles based on CMC and GEL crosslinked with EpCl can be classified as low toxicity products, recommended for biomedical applications.

Obtained particles are, from the diameter point of view, between the limits of 1.4 and 64.5 μm. This gives them the possibility to be used in medical applications as sprays, designed for treatment of airways diseases (by inhalation) or for application on mucous membrane (nose, vagina, rectum), or on the skin.

The biologically active chemical to be used for the inclusion and the release is cefotaxime (CFTS), antibiotics indicated for serious disease (with

germs sensitive to CFTS) with different positions: air or genito-urinary ways, intra-abdominal infections… Particles selected as inclusion support have diameters comprised between 1.4 and 6.3 μm, ensuring the targeting of lung, terminal bronchial or nasal tubes respectively.

The drug inclusion was carried out by diffusion process of the aqueous solution towards the particles. From the kinetic point of view, the drug inclusion was rapid and the equilibrium was obtained in less than 120 minutes. The maximal quantity of CFTS, at equilibrium, included within the microparticles and released from them is influenced by the swelling capacity of the particles in aqueous media which depends on different factors such as the crosslinking density of the network, the composition and the size of the particles. A direct proportionality was found between the maximal swelling ratio and the CFTS inside the particles, whatever the microparticles series, respectively the studied parameter. The maximum amount of included CFTS varies, for all series of particles, between 6.2 and 18.6 mg_{CFTS}/g particles.

By correlating the quantity of released drug with some parameters of particles synthesis (Table 2) it was found: **(i)** the CFTS fraction released by polymer-drug system is between 89.5 and 97.5% **(ii)** the fraction of included drug changes as the polysaccharide content in the particles, or even the maximal swelling ratio **(iii)** only for particles obtained with a variable r_{PE} the fraction of released drug correlates the size and the CMC content of particles.

The variation of the drug released with time is shown in Fig. 10 for four series of particles.

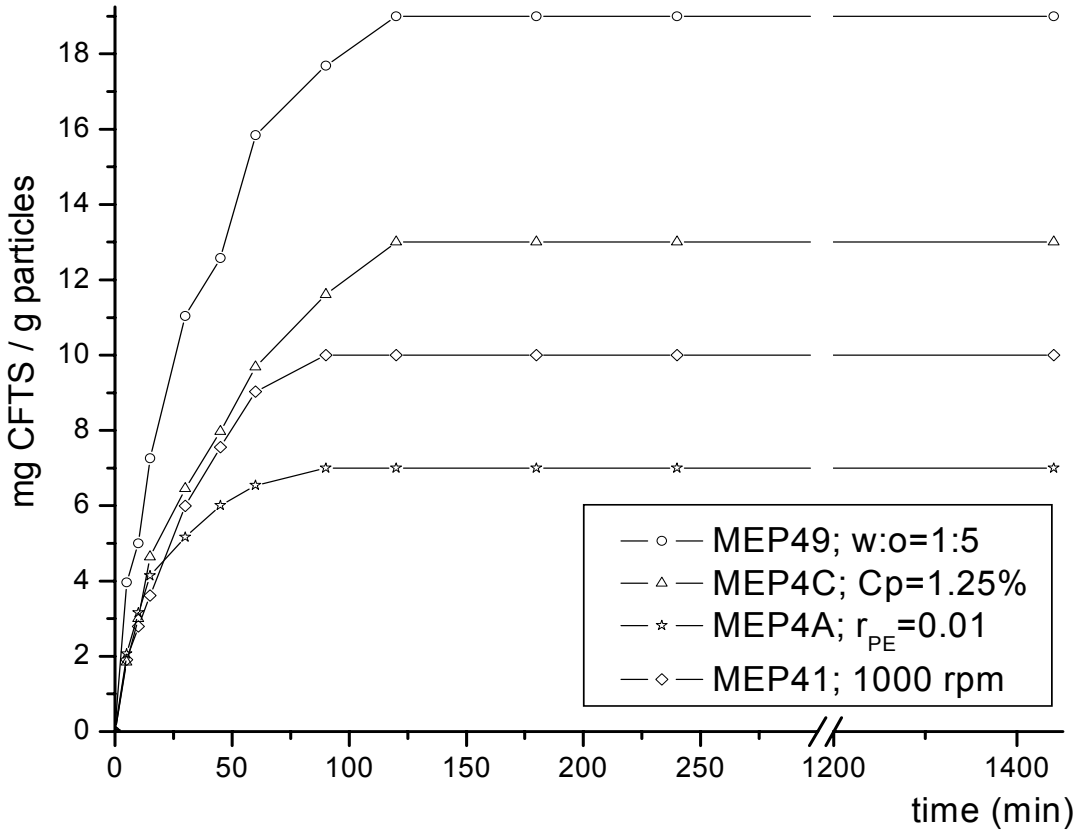

Fig. 10 – Inclusion kinetics of CFTS for microparticles those diameters vary between 1.4 and 6.3 μm.

The release process reaches the equilibrium after at maximum 60 minutes and it is quicker than the inclusion. The quick release is due to the CMC present in the particles because repulsion electrostatic forces exist between carboxylate groups coming from CMC and the drug (sodium cefotaximate).

Antibacterial activity of drug loaded particles

Antimicrobial activity of MEP4 particles loaded with cefotaxime was evaluated on two bacterial cultures, negative Grams (*Pseudomonas aeruginosa* ATCC 27853) or positive Grams (*Staphylococcus aureus* ATCC 25923). Analysis of results shows

that the polymer-drug system keeps its antibacterial activity determined by the drug. Free-drug microparticles do not develop inhibition zones. Moreover, the polymer-drug system keeps its antibacterial activity for a long time (48 hours).

EXPERIMENTAL

Materials

Carboxymethylcellulose (CMC) (sodium salt) – FLUKA**:** DS ≈ 0.752, Mw ≈300 000 g/mol.

Gelatin (GEL) type A – MERCK: $\bar{M}$ =100.000Da, pH_{is}=6.73, intrinsic viscosity 2.7 dL/g.

Epichlorohydrin (EpCl) – ALDRICH: M=92.53 g/mol, ρ=1.181 g/mL.

Tween 80 (non ionic surfactant) – ALDRICH (9005-65-6): HLB=15.0, M_w=1310, ρ=1.08 g/mL.

Brij 52 (non ionic surfactant) – CRODA (610A0804): HLB=5.3, M_w=330, ρ=0.978 g/mL.

Cefotaxime (CFTS) – water soluble antibiotics (sodium salt).

Preparation of polymer particles

Polymeric particles were synthesized using a reverse emulsion (w/o) process from the dispersion of the aqueous phase (hydrophilic surfactant and polymers to be crosslinked) within an immiscible organic phase (toluene). To stabilize the emulsion whose HLB is equal to 6.5, a mixture of surfactants was used. It was composed of a hydrophobic surfactant (Brij 52, HLB=5.3) and an hydrophilic one (Tween 80, HLB=15). The surfactant mixture concentration (C_t) was 0.15% (g/mL of emulsion), and the hydrophilic-hydrophobic ratio was calculated from the relation given in the literature.[27, 28]

The hydrophobic surfactant solution was prepared in a three-necked reactor with a mechanical stirring. [29,30] The hydrophilic surfactant was dissolved in the aqueous polymer solution (weight CMC/GEL ratio = 1.5 g/g) which pH was adjusted at a value of 10 with a 40% sodium hydroxide solution. This solution was introduced within the reactor under a continuous stirring, at 800 rpm. After 30 minutes, when the dispersion is homogeneous, whitish and more viscous, the crosslinking agent was added, this instant being measured as time zero of the reaction. At the end of the reaction the emulsion was broken by centrifugation (10 minutes, 6000 rpm). The organic phase was removed and the microparticles were submitted to several washing-separation cycles by centrifugation using alternately water and acetone to remove products which have not reacted, by-products and toluene traces. After the last acetone washing the particles were dried first in an oven at 40°C and finally under vacuum during 24 h at 20°C. Table 2 shows the experimental program.

Table 2

Experimental program for obtaining particles based on CMC and GEL crosslinked with EpCl

Code sample	Polymers mixture /crosslinking agent ratio, r_{PE} (g/g)	Crosslinking time, t_R (h)	Conc. of polymers solution, Cp (%)	Hydrodymamic regime (rpm)	Aqueous phase/ organic phase ratio, w:o (v/v)	Fraction of released drug $CFTS_{released}/CFTS_{included}$ (%)
MEP4A	0.08	4	2	700	1:4	94
MEP4	0.1					94.4
MEP4B	0.16					95.5
MEP42	0.1	2	2	700	1:4	-
MEP43		3				-
MEP4		4				94.4
MEP45		5				-
MEP4C	0.1	4	1.25	700	1:4	-
MEP4D			1.50			-
MEP4			2			94.4
MEP4E			2.25			92
MEP46	0.1	4	2	600	1:4	94.4
MEP4				700		94.4
MEP41				1000		95.5
MEP49	0.1	4	2	700	1:5	97.5
MEP4					1:4	94.4
MEP48					1:3	92.5
MEP47					1:2	89.5

Analysis

FTIR spectroscopy – **DIGILAB Scimitar FTS 200** spectrometer–USA, KBr pellet.

NMR spectroscopy RMN – **Bruker Advance** spectrometer (400,13 MHz), ^{1}H-HRMAS technique with D_2O at 25°C.

Scanning Electron Microscopy – **ElectroScan E3** microscope (20 kV).

Size analysis and size polydispersity of microparticles – laser diffraction analyzer (purple, λ = 405 nm) **Shimadzu – SALD 7001.** The microparticles were suspended in acetone to avoid their swelling, leading to sizes close to dry state. A series of fives analysis were performed on each sample.

Analysis of the composition – the gelatin content was determined from the nitrogen evaluation using Kjeldahl method;[31] CMC content was measured from the transformation of sodium in sodium sulfate and calcination at 600°C followed by gravimetric determination.

Swelling ratio – Maximal swelling degree was measured by thermogravimetry. Temperature was increased according to a continuous ramp (10ºC/min) from 30 to 120ºC, followed by an isotherm at 120ºC during 60 minutes, in air (thermobalance **TA Instruments 2950**). Samples were first immersed within water during pre-determined durations. Then the excess of water was removed by soft swabing with a filter paper. Swollen particles were placed inside the aluminum crucible before beginning the temperature program and recording of the thermogram from which the swelling degree was calculated as ratio between the amount of absorbed water and the amount of dried particles under analysis (%).

Inclusion and release of drugs in/from microparticles

Inclusion and release of drugs were carried out by diffusion of its aqueous solution within/from microparticles. 0.05 g of microparticles beforehand swollen up to equilibrium were suspended in 50 mL of the aqueous drug solution at a known concentration. After pre-determined time intervals 0.5 mL were removed; after dilution up to 10 mL the absorbance was measured (**Helios Alpha** spectrometer– England) at the specific wavelength of maximal absorbance of the drug. From the calibration curves the drug concentration of the supernatant was measured.

To study the drug release kinetics, loaded microparticles were centrifuged at 6000 rpm and sediment was transferred in 50 mL of twice-distilled water. Periodically 0.5 mL were removed; after dilution up to 10 mL the absorbance was measured.

Toxicity of particles

Toxicity was evaluated from the Speerman-Karber method.[32] Tests were performed on rats which weight was 20±2g.

CONCLUSIONS

Interpenetrated network type microparticles based on natural polymers (carboxymethylcellulose and gelatin) were elaborated using an inverse emulsion crosslinking process. The composition, the morphology, swelling in water characteristics, the capacity to include and release water soluble drugs of these particles depend on elaboration process parameters such as: the w/o ratio, the hydrodynamic regime or the polymer mixture/crosslinking agent ratio. The ability to include drugs is directly correlated with the swelling degree, the gelatin content within the particles and the pH of the swelling. Polymer-drug systems elaborated under particles keep their bactericide activity during at least 48 hours. The absence of toxicity, associated with the bactericide activity, make these systems potential drug carriers.

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