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Phosphorylated curdlan microgels. Preparation, characterization, and in vitro drug release studies

Irina Popescu[a], Irina M. Pelin[a], Maria Butnaru[b], Gheorghe Fundueanu[a], Dana M. Suflet[a,*]

[a] Petru Poni Institute of Macromolecular Chemistry, Aleea Grigore Ghica Voda 41A, 700487 Iasi, Romania
[b] Gr.T. Popa University of Medicine and Pharmacy, Faculty of Medical Bioengineering, Department of Biomedical Science, Str. Kogalniceanu 9-13, Iasi, Romania

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ABSTRACT

Curdlan derivative with anionic phosphate groups was used for the first time to obtain hydrogel microspheres. The chemical cross-linking of the phosphorylated curdlan was performed with epichlorohydrin using the water-in-oil inverse emulsion technique. The optical and scanning electron microscopies were used to analyze the morphology of the microgels, whereas the FTIR spectroscopy was used to investigate their chemical structure. The main characteristics such as the swelling degree, the exchange capacity, and the thermal resistance were also studied. These new anionic microgels could be used as potential carriers for controlled release of opposite charged drugs retained through electrostatic forces. Diphenhydramine, a cationic model drug, was used to investigate the loading and the release processes in various pH media simulating physiological fluids. Several mathematical models were applied to evaluate the drug transport processes and to calculate the drug diffusion coefficients. The synthesized microspheres presented an excellent biocompatibility.

1. Introduction

Microgels are cross-linked hydrogel particles of small dimensions having hydrophilic character, porous structure, adjustable chemical and mechanical properties, an interior network for the incorporation of therapeutic agents, and are often biocompatible (Oh, Lee, & Park, 2009). These unique properties offer a great potential for their use in different pharmaceutical and cosmetic formulations, drug delivery systems, biomedical implants, or in tissue engineering (Coviello, Matricardi, Marianecci, & Alhaigue, 2007; Hoffman, 2002). In addition, the microgels can be defined as materials that exhibit the ability to swell in water due to the presence of the hydrophilic groups such as hydroxyl, amine, carboxyl, sulfate, etc. Due to their structural features, these microgels can be mainly prepared through the chemical and/or physical cross-linking of polymers. The chemical cross-linking is a highly versatile method of preparing hydrogels with good mechanical stability (Oh et al., 2009). Using the chemical cross-linking in suspension or emulsion, spherical particles with diameters ranging from 1 μm to 1 mm could be obtained (Liu, Jiao, Wang, Zhou, & Zhang, 2008; Oh, Drumright, Siegwart, & Matyajaszewski, 2008; Oh et al., 2009; Peppas & Khare, 1993).

Biomicrogels – microgels based on biopolymers – have the property of being biocompatible, non-toxic, and biodegradable. Typical examples of biopolymers are the polysaccharides (chitosan, hyaluronan, dextran, microbial cellulose, pullulan, alginate, and their derivatives) known as being non toxic, renewable resources, and having a relatively low manufacture cost. A large number of studies regarding the use of microgels based on polysaccharide as drug delivery systems have been reported in literature, but a low interest has been attributed to curdlan (Coviello et al., 2007; Liu et al., 2008). Curdlan is a bacterial polysaccharide resulting from pure culture fermentation of *Agrobacterium biobar 1*, with a linear structure composed entirely of D-glucose units linked by β-(1 → 3) glucosidic bonds (Laroche & Michaud, 2007). Curdlan is soluble in diluted base solutions (0.25 M NaOH), dimethylsulfoxide (DMSO), and formic acid, but it is not soluble in alcohols or in water due to the existence of extensive intra/intermolecular hydrogen bonds. The current applicability of the curdlan is still limited due to its insolubility in water, but this drawback can be eliminated by the introduction of ionic groups. A limited number of papers regarding the synthesis of the curdlan derivatives with ionic groups such as the sulfate (Fritzsche et al., 2006), phosphate (Chen, Xu, Zhang, & Zeng, 2009; Suflet, Nicolescu, Popescu, & Chitanu, 2011), carboxymethyl (Gao et al., 2008), and ammonium groups (Sugikawa, Numata, Kaneko, Sada, & Shinkai, 2008) have been reported. The curdlan and its derivatives have a high potential for pharmaceutical applications such as the delivery systems for proteins, drugs, enzymes, etc. (Chen & Seviour, 2007). Moreover,

* Corresponding author. Tel.: +40 232 217454; fax: +40 232 211299.
E-mail address: dsuflet@icmpp.ro (D.M. Suflet).

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the curdlan and its sulfate derivatives proved to have an important biological activity such as the anti-tumour, anti-HIV (Borjihan, Zhong, Baigude, Nakashima, & Uryu, 2003; Jeon et al., 2000; Kaneko et al., 1990; Osawa et al., 1993; Yoshida et al., 1995), anti-coagulant (Alban & Franz, 2001), anti-oxidant activity, or immunomodulatory effects (Chen & Seviour, 2007; Laroche & Michaud, 2007). Some papers reported the synthesis of the support matrices for the enzyme immobilization (Saudagar & Singhal, 2004) or of the gels/microparticles as carriers for protein/vaccine/enzyme/drug (Kim et al., 2000; Mocanu, Mihai, Moscovici, Pictonc, & LeCerf, 2009) using the non ionic curdlan, but the ionic groups introduced by subsequent reactions are distributed especially to the surface of the particles. A more uniform distribution of the ionic groups could be obtained by the cross-linking of an ionic curdlan derivative, but according to our knowledge the data concerning the synthesis of such microgels have not been reported in the literature.

In this paper, new anionic microgels based on monobasic curdlan phosphate were obtained by chemical cross-linking with epichlorohydrin using the water-in-oil inverse emulsion technique. The morphology of the microgels was analyzed by optical and scanning electron microscopy and the chemical structure was confirmed by FTIR spectroscopy. The main characteristics of these new microgels such as the swelling degree in various pH solutions and the exchange capacity were determined. The thermal stability and the biocompatibility were also investigated. These new anionic microgels could be used as potential carriers for drug delivery and the uniform distribution of the ionic groups in the microgels structure could lead to a retardation of the drug release. In order to verify this supposition, the microgels were loaded with diphenhydramine hydrochloride, which was taken as a model drug and the release profiles were studied in various pH media to simulate the physiological body fluids (pH = 7.4 from duodenum intestinal fluid and pH = 1.2 for gastric juice). To facilitate the product development in the future and to help the understanding of the complex pharmaceutical dosage forms, several mathematical models were applied to evaluate the drug transport processes from these new microgels and its diffusion coefficients were calculated.

2. Materials and methods

2.1. Materials

Curdlan (Curd) (Wako Pure Chemical Ind., Japan), epichlorohydrin (ECH), 1,2-dichloroethane, cellulose acetate butyrate (CAB), sodium hydroxide, acetone, and ethylic ether (Sigma–Aldrich, Germany) were used as received. Monobasic curdlan phosphate (PCurd) (with a degree of substitution up to 1) was synthesized in our laboratory using a method which was described elsewhere (Suflet et al., 2011). Diphenehydramine hydrochloride (DPHAH) (Sigma, Germany) was taken as a model drug. All experiments were performed using twice-distilled water. Dulbecco's modified Eagle's media (DMEM) with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, stabilized penicillin/streptomycin/neomycin (PSN) solution, bovine foetal serum (BFS), and Dulbecco's phosphate buffered saline (DPBS) were also purchased from Sigma–Aldrich, Germany for biological tests.

2.2. Methods

2.2.1. Synthesis of microspheres

Monobasic curdlan phosphate microgels (PCurdMG) and curdlan microgels (CurdMG) were prepared by chemical cross-linking with ECH using the water-in-oil (w/o) inverse (mini)emulsion technique as previously reported (Nastruzzi et al., 1993). The microgels were obtained using a cylindrical glass reactor equipped with an anchor type glass stirrer, and a reflux condenser. Briefly, 2 g of polysaccharide (monobasic curdlan phosphate or curdlan) were dissolved in 0.5M NaOH aqueous solution (40 ml). The solution was poured in 100 mL of dispersion medium (1,2-dichloroethane) in which 3 g of CAB (as dispersion agent) were dissolved. The w/o emulsion was stirred for 1 h at 700 rpm for the obtaining of a stable dispersion, then 10 mL of ECH were added and the cross-linking reaction was carried out at 55 °C for 96 h. The cross-linked microgels were recovered by filtration and then washed for the removal of residuals in the following order: 1,2-dichloroethane, acetone, twice-distilled water, acetone, and ethylic ether. Finally, the microgels were completely dried by overnight exposure to 60 °C, under vacuum.

2.2.2. Analysis and characterization of curdlan phosphate microgels

FTIR spectra were recorded on KBr pellet using a Vertex 70 Bruker spectrometer. The microgels morphology was evaluated by optical microscopy and scanning electron microscopy (SEM) observations. The microparticles were sputter-coated with gold prior to the acquisition of SEM images, using a (SEM-EADX) Quanta 200 (FEI) electron microscope. The wrack density was determined by weighing the volume of 1 mL of dried microgels placed in a graduated cylinder (i.d. = 11 mm). The swelling degree was evaluated by determining the volume expansion of microgels at equilibrium placing the dried microgels in different buffer solutions, with the same ionic strength, such as pH 1.2 (HCl + KCl), phosphate buffer solution at pH 7.4 ($NaH_2PO_4 + Na_2HPO_4$) and twice-distilled water at pH 5.5 (adjusted with NaOH or HCl). The swelling factor (q), defined as the volume of the swollen microgels (V_s) related to the dry volume (V_d), was measured by placing the dried microgels in a graduated cylinder (i.d. = 11 mm).

The exchange capacity of the microgels (EC) was determined by titration method, using a Metrohm all purposes 716 DMS Titrino apparatus equipped with a 6.0203.100 combined electrode. Firstly, 100 mg of ionic microspheres were swollen in 20 mL water for 12 h. After that, an excess of 0.1 N HCl solution (5 mL) was added and maintained for 5 h, under gentle stirring. The supernatant solution was collected and titrated with a 0.1N NaOH solution. The exchange capacity (EC) was expressed as meq. phosphoric groups/g dried microgels.

The thermal decomposition under dynamic conditions of heating was investigated with a Paulik–Paulik–Erdey MOM-Budapest instrument on 50 mg samples, at 12 °C/min, in air.

2.2.3. Drug loading

The microgels with the phosphate groups in Na^+ form were previously treated with 0.1N HCl solution, to convert them into acid form. Then, 100 mg of microgels in H^+ form were immersed in 20 mL aqueous solution containing a model drug, DPHAH (1 mg/mL) and kept for 24 h at room temperature. The excess of the drug, related to the total EC of microspheres was 2:1. Finally, the microgels were filtered, washed with twice-distilled water in order to remove the excess of drug, and then dried using the water–acetone mixtures in the various volume ratios. The amount of the retained drug was determined by the UV method at $\lambda = 218$ nm, using a Specord 200 Analytic Yena UV–vis spectrophotometer. Previously made calibration curves were used. The drug loading was calculated according to Eq. (1).

$$\text{Drug loading} \quad (\%) = \left(\frac{Q_d}{Q_m} \times 100 \right) \tag{1}$$

where Q_d is the weight of drug entrapped in microspheres, and Q_m is the weight of the dry microspheres.

2.2.4. In vitro drug release

The in vitro drug release studies were performed through the bath method (Zografi, Scott, & Sworbrick, 1990) using twice-distilled water (pH 5.5) or different buffer solutions (pH 7.4 and 1.2), with and without added salt, simulating the physiological fluids. The loaded microparticles samples (100 mg) were immersed in the release medium (100 mL) under gentle stirring at 37 °C (±0.1 °C). At predetermined time intervals, the solution samples were withdrawn and the drug content was determined by spectrophotometric method. The same volume of the fresh buffer was added to replace the volume of the withdrawn samples. Each experiment was conducted in triplicate.

The analysis of the drug release kinetics from PCurdMG was performed by calculating the diffusion coefficients, D_E and D_L, using the early-time (Eq. (2)) and the late-time (Eq. (3)) equations, respectively. These equations are approximations of the equation that is obtained when solving Fick's second law of diffusion under initial and boundary conditions (Ritger & Peppas, 1987a; Siepmann & Siepmann, 2008).

$$\frac{M_t}{M_\infty} \cong 4\left(\frac{D_E t}{\pi\delta^2}\right)^{0.5} \tag{2}$$

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \quad \exp\left(-\frac{\pi^2 D_L t}{\delta^2}\right) \tag{3}$$

where M_t and M_∞ are the absolute amount of drug released at t and infinite time, D_E and D_L are the corresponding diffusion coefficients and δ is the diffusion distance (the radius of the particles).

2.2.5. Mathematical analysis of the drug transport mechanism

In order to study the DPHAH transport mechanism from loaded PCurdMG, five diffusion models were considered to fit the experimental data. These models were originally developed to describe the release of solutes from polymeric devices with different shapes (slabs, spheres, cylinders or discs). *Model 1* was described by Ritger–Peppas equation (Dash, Murthy, Nath, & Chowdhury, 2010; Ritger & Peppas, 1987a, 1987b):

$$\frac{M_t}{M_\infty} = k_1 \cdot t^n \tag{4}$$

where M_t and M_∞ are the absolute amount of drug released at t and infinite time, respectively; k_1 is the constant characteristic of the drug-polymer system, and n is the diffusion exponent characteristic of the drug transport mechanism. According to the criteria for release kinetics from swollen spherical systems, the release exponent values indicate: a Fickian diffusion mechanism when $n \leq 0.43$; an anomalous (non-Fickian) drug transport mechanism when $0.43 < n < 0.85$; a Case-II transport leading to zero-order diffusion when $n = 0.85$ and a super-Case-II transport of the drug when $n > 0.85$.

Model 2 based on the Peppas–Sahlin equation (Eq. (5a)) with the m exponent fixed to 0.5 (Eq. (5b)) could also be used to describe the release behaviour of dynamically swelling hydrogels:

$$\frac{M_t}{M_\infty} = k_1 t^m + k_2 t^{2m} \tag{5a}$$

$$\frac{M_t}{M_\infty} = k_1 t^{1/2} + k_2 t \tag{5b}$$

where the first term of the equations represents the contribution of Fickian diffusion and the second term refers to the macromolecular relaxation contribution to the overall release mechanism. Using the estimated parameters k_1 and k_2 obtained from fitting Eq. (5b), the ratio of relaxation (R) and the diffusional (F) contributions were calculated using Eq. (6):

$$\frac{R}{F} = \frac{k_2}{k_1} t^{1/2} \tag{6}$$

Model 3 represents a zero-order drug release which is expressed by the following equation:

$$\frac{M_t}{M_\infty} = k_d t \tag{7}$$

where k_d is the kinetic dissolution constant.

Model 4 is based on Higuchi equation and describes the Fickian diffusion of a drug from an insoluble matrix (Serra, Domenech, & Peppas, 2006):

$$\frac{M_t}{M_\infty} = k_H t^{1/2} \tag{8}$$

where k_H is a kinetic constant.

Model 5 is an interesting semi-empirical model proposed by Hopfenberg (Siepmann & Siepmann, 2008) for a quantitative description of drug release from degradable drug delivery systems exhibiting a release rate which is proportional to the surface area of the device (Eq. (8)). In this model, all mass transfer processes that are involved in the control of drug release are assumed to add up to a single zero order process (characterized by a rate constant, k_0).

$$\frac{M_t}{M_\infty} = 1 - \left(1 - \frac{k_0 t}{c_0 a}\right)^n \tag{9}$$

where c_0 denotes the uniform initial drug concentration within the system; a is the radius of the sphere or cylinder or the half-thickness of the slab; and n is a shape factor representing spherical ($n = 3$), cylindrical ($n = 2$) or slab geometry ($n = 1$). The model ignores edge and end effects.

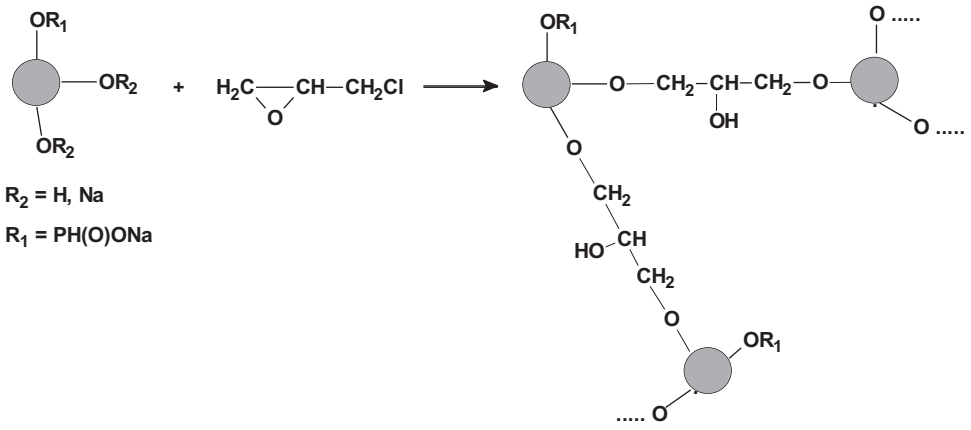
These mathematical models are valid only for the first 60% of the total amount of drug release. To distinguish the models that described the data properly from those that did not fit the data correctly, the sum of the squared residuals (SSR) was calculated. The model that best explains the experimental data is the one that shows the minimal value of SSR. However, since a large number of model parameters could lead to a higher probability of obtaining a smaller SSR value, it was necessary to use a discriminatory criterion that was independent of the number of parameters that each model had. For this reason, the Akaike Information Criterion (AIC) was applied, defined as:

$$\mathrm{AIC} = N(\ln\ SSR) + 2p \tag{10}$$

where N is the number of experimental data points, SSR the sum of the squared residuals, and p the number of parameters. The model that shows the smallest value for AIC is the one which statistically best describes the drug release mechanism.

2.2.6. Biocompatibility test

The biocompatibility of the CurdMG and PCurdMG was analyzed based on morphological assay of primary rat dermal fibroblasts after their direct contact with the swollen microspheres in the culture medium. The rat dermal fibroblasts (RDFs) were isolated according to literature (Ian Freshney, 2005). Briefly, 1.5 mg of dried microspheres of CurdMG or PCurdMG were sterilized with 1 mL of 70% ethanol solution, for 20 min. The sterilized microspheres were separated from ethanol solution by straining through nylon mesh with a pore size of 40 μm. The washing procedure was done sequentially in DPBS and DMEM media. After washing, the microspheres were swollen in DMEM media, at 37 °C and 5% CO_2 atmosphere for 24 h. The suspension of the swollen microspheres was equally split in the culture plate wells and a concentration of 40,000 RDF cells/well was added and incubated for 24, 48, 72 and 192 h. The DMEM medium was changed at 72, 120, and 168 h. The microscopic images of the living cells were done at each incubation point, using Olympus inverted microscope. 192 h were chosen as end point for cell fixing and staining because at that time the maximum cells density was observed. Briefly, the cells grown in the



Scheme 1. Synthesis of the CurdMG and PCurdMG by chemical cross-linking with epichlorohydrin.

presence of the microspheres were washed with DPBS, fixed for 10 min with Bouin's solution, washed with distilled water and then stained using haematoxylin-eosin (HE) procedure (Lynch, Raphael, Mellor, Spare, & Inwood, 1969). The stained cells were analyzed using Olympus inverted microscope and an additionally provided camera.

3. Results and discussion

3.1. Preparation and characterization of CurdMG and PCurdMG

The microgels based on neutral and phosphorylated curdlan were synthesized by chemical cross-linking using the w/o inverse emulsion technique. The chemical structure of the microgels obtained by the cross-linking of the ionic and non-ionic polysacharides with ECH is presented in Scheme 1.

The synthesis of the microgels by the cross-linking reaction was carried out at various reaction times using the PCurd with different degrees of substitution (DS). The well-defined microgels were obtained after 96 h only for phosphorylated curdlan with DS up to 1. Low reaction times led to the obtaining of non-defined and unstable microgels. The formation of microgels was not possible when DS > 1, because of the reduced number of hydroxyl groups that could participate to the cross-linking reaction. Also, at DS > 1, the density of voluminous phosphorylated groups was high, hindering the access of the cross-linked agent to the reaction sites. The chemical structure of the ionic/non-ionic microgels was confirmed by FTIR spectroscopy (see Supplementary Data).

The size and the morphology of the microgels were investigated by optical microscopy and SEM. The spherical structure of the curdlan and curdlan phosphate microgels in hydrated state is shown in Fig. 1a and b.

The SEM micrographs of the microgels in dehydrated state are presented in Fig. 1c and 1d. These images confirm that the cross-linking degree and the ionic charge of the polymer play an important role in the surface morphology of the formed microparticles (Zhao & Qiu, 2011). In the case of non-ionic CurdMG, the particles surface is smooth with small pores. On the other hand, in the case of the curdlan with phosphoric groups, a wrinkled surface of the microparticles is observed. This major difference between the microparticles morphology is due to the cross-linking degree, which is higher for the non-ionic curdlan (with three free hydroxyl groups) compared with the ionic curdlan with only two free positions. In Fig. 1e the size distribution of the ionic/non-ionic microspheres is presented. Most of the particles have the size between 60 and 90 μm, the PCurdMG having a narrower size distribution.

The main characteristics of PCurdMG and CurdMG such us wrack density, exchange capacity (EC), and swelling degree in various pH solutions are presented in Table 1. The higher cross-linking degree for CurdMG compared to PCurdMG is reflected in the main characteristics: higher wrack density and lower swelling degree.

Obviously, the non-ionic microgels are not sensitive to the pH, the degree of swelling being approximately the same in different media. When ionic groups are present in microgels (PCurdMG), the swelling degree is influenced by the pH of the medium and by the ionic strength. It can be observed that the swelling degree is much higher in water, compared with that in buffer solutions. In water (without ionic strength), the chains of phosphorylated curdlan are extended due to the electrostatic repulsion between the —PH(O)O$^-$ groups, leading to the swelling of the microgels, while at high ionic strength (buffer solutions) the electrostatic repulsions are shielded. In acidic buffer solution (pH = 1.2) the phosphate groups from the polymer chains (with pK_a = 2.8) are not dissociated and the swelling degree is low. As expected, in phosphate buffer solution with pH = 7.4, where the phosphoric groups are ionized, the swelling degree is higher.

The obtained microspheres could be used for the retention of various active principles with applications in pharmaceutical/medical field. For this aim, the microspheres must undergo the sterilization process, at 120–130 °C. In this respect, the thermal decomposition under dynamic conditions of heating was investigated (Fig. 2).

The thermal decomposition proceeds in two main steps. In the temperature range 80–220 °C, CurdMG and PCurdMG exhibit a 10% weight loss that can be attributed to the evaporation of the adsorbed moisture. The next steps at temperatures higher than 220 °C can be attributed to the oxidative decomposition of the polymers. The total loss of CurdMG is 96% while PCurdMG presents a total loss of 88%. This behaviour could be explained by the presence of phosphoric groups that probably led to the formation of polymethaphosphoric acid during the decomposition process, which inhibits the further decomposition. The thermal analysis proves the stability of microspheres at 110–130 °C, which is useful for further applications when sterilization is compulsory.

3.2. Loading of DPHAH on microspheres

DPHAH was chosen as a model drug for the loading and release experiments, because it possesses strong tertiary amino groups able to interact electrostatically with the negatively charged PCurdMG. The amount of the DPHAH entrapped in neutral charged CurdMG is very low due to a weak retention which occurs only by physical forces. In this case, the adsorbed drug has been

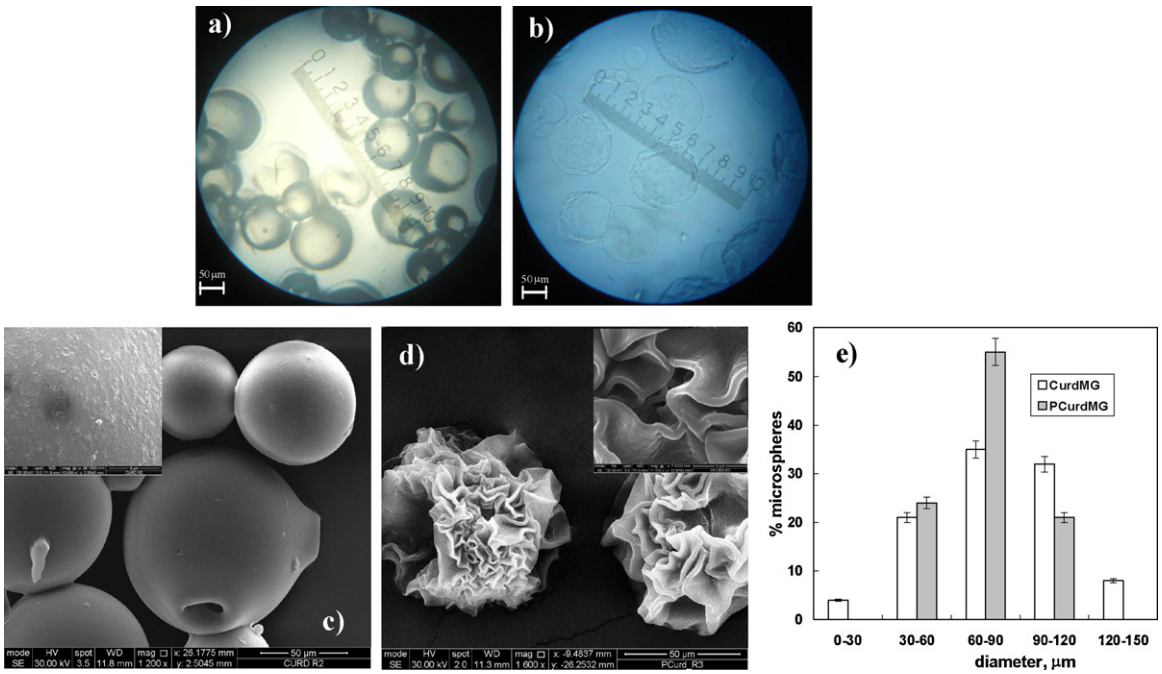


Fig. 1. (a) Optical micrographs of CurdMG and (b) PCurdMG in hydrated state. (c) Scanning electron micrographs of CurdMG and (d) PCurdMG in dry state and their details. (e) The particles size distribution.

Table 1
Main characteristics of curdlan and phosphorylated curdlan microgels[a].

Sample code	Wrack density, g/mL	EC, mequi./g	Swelling degree, *q*		
			Water	pH = 1.2	pH = 7.4
CurdMG	0.69 ± 0.02	–	7.0 ± 0.18	5.0 ± 0.15	5.0 ± 0.15
PCurdMG	0.53 ± 0.02	0.43 ± 0.01	50.0 ± 0.15	23.5 ± 0.10	45.6 ± 0.15

[a] The values are the mean of three-independent measurements ± SD.

easily removed during the washing step of the microgels. The drug loading is found to be 0.017 ± 0.2% (w/w) for the non-ionic microgels (CurdMG) and about 4 ± 0.15% (w/w) for the anionic microgels (PCurdMG). The efficiency of the drug inclusion for PCurdMG (calculated taking into account the actual amount and the theoretical amount of retained drug based on EC) is around 33%. The relative low loading degree suggests that the large molecules of drug hinder, after linking, the access of the new molecules to the vacant binding sites.

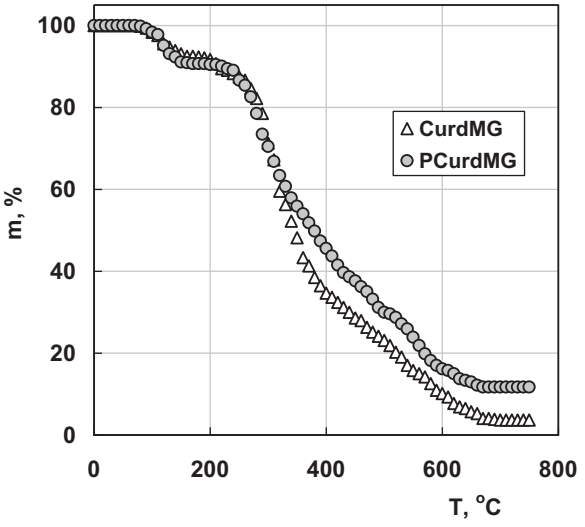


Fig. 2. Thermogravimetric curves of curdlan and phosphorylated curdlan microgels.

3.3. Drug release studies

In vitro release studies give important information on the efficiency of a delivery system. Release studies were performed under pseudo-physiological conditions or in pure water or aqueous solutions with different ionic strength. As it is known, for the drug delivery systems in which the drug is ionically linked, the release of the drug is controlled firstly by the rate of cleavage of electrostatic bonds and secondly by a diffusion process. In our case, the ionic groups of the curdlan phosphate have a moderately strong acid character (Suflet et al., 2011). On the other hand, the DPHAH possesses strong basic character, so it is expected to form stable complexes. As shown in Fig. 3a, the amount of the drug released in pure water is relatively low, even after 100 h, and represents the drug physically entrapped. The release of the drug occurred gradually and presents three parts. In the first part, 5 h, no drug is released, because the microspheres are still unswollen. The second part is characterized by a rapid diffusion of the entrapped drug from the peripheral regions of the microspheres and in the last part the release is controlled mainly by diffusion. In the presence of 0.15 M NaCl, the electrostatic interactions between the phosphate groups and the drug molecules were screened by the added salt. In this case, the breaking of the ionic interactions has a higher contribution to the drug release rate than the diffusion and the swelling processes.

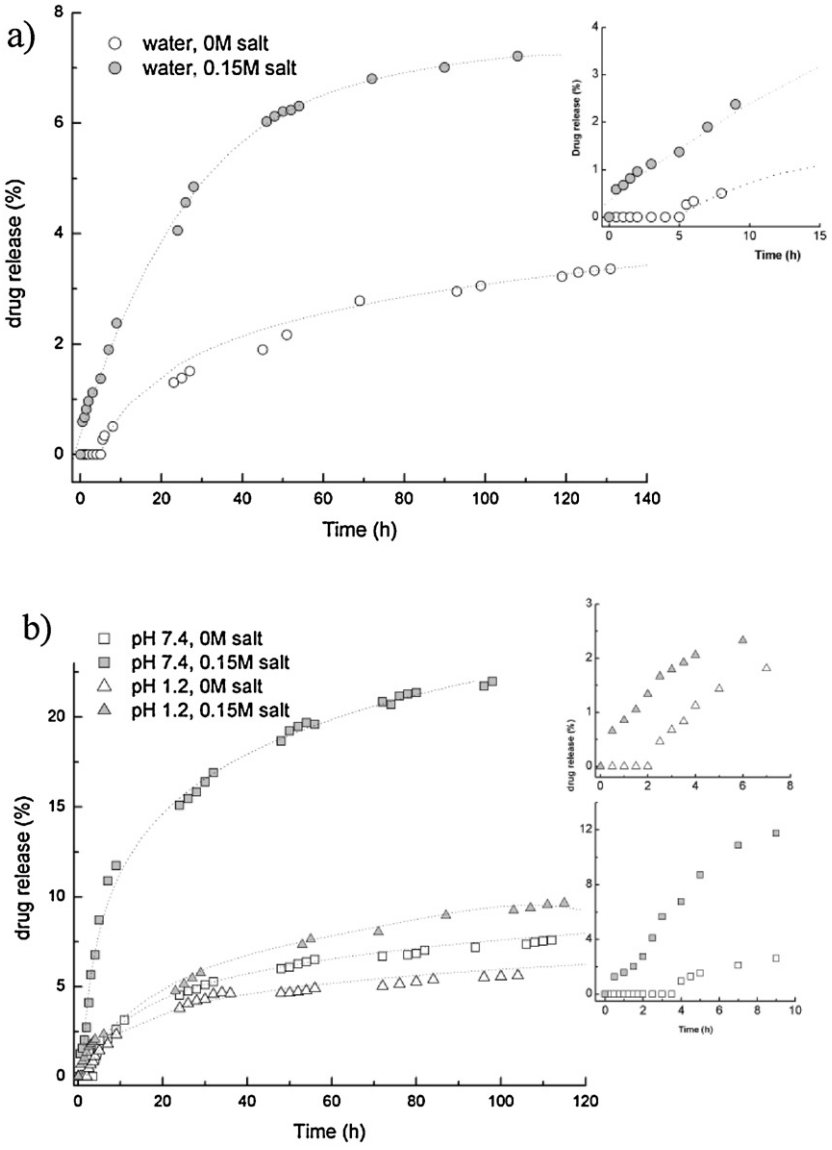


Fig. 3. Drug release profiles of DPHAH from PCurdMG in water (a) and different buffer solutions (b) with and without added salt.

In order to study the drug release in physiological fluids, different buffer solutions simulating the intestinal fluid (pH = 7.4) or gastric juice (pH = 1.2) were used. The release profiles of the drug in the buffer solutions with and without added NaCl are presented in Fig. 3b. In acidic conditions (pH = 1.2), the amount of drug released from PCurdMG, as well as the release rate are lower than in buffer solution at pH = 7.4. This behaviour could be attributed to the fact that at acidic pH the phosphorous groups are in protonated form ($pK_a = 2.8$), less hydrophilic, while in basic condition these groups are dissociated, and the microgels swell more (see Table 1) (Fundueanu et al., 2005; Singh, Sharma, & Chauhan, 2007).

The intimate mechanism of swelling/drug release in the presence of the free ions from buffer or added salts is explained. When the ionic strength is increased due to the ions of the buffer solution, the microgels can exchange ions with the release medium. Thus, the microgels maintain the charge neutrality, and the concentration of free counter ions inside the microgels will increase. An osmotic pressure difference will arise between the microgels and the release solution, and will cause the microgels to swell (Bajpai, Shukla, Bhanu, & Kankane, 2008). When the ionic strength is increased to high level by adding the 0.15 M NaCl, the microgels shrink, and a drug burst release is observed (Fig. 3b). A delay in drug release in the absence of added salts should be emphasized. In fact, these salts induce initially a rapid swelling of hydrogels followed by the release of the drug.

Data obtained from the release studies were fitted to the early-time and the late-time approximation equations (Eqs. (2) and (3)), and the corresponding diffusion coefficients D_E and D_L were calculated. These diffusion coefficients represent a measure for

Table 2
Diffusion coefficients of diphenehydramine hydrochloride.

pH medium	Early-time approximation eq.		Late-time approximation eq.	
	$D_E \times 10^{11}$ ($cm^2\ min^{-1}$)	R^2	$D_L \times 10^{11}$ ($cm^2\ min^{-1}$)	R^2
7.4, 0 M salt	0.541	0.948	0.00428	0.992
1.2, 0 M salt	0.224	0.954	0.4280	0.926
Water, 0 M salt	0.044	0.964	0.2860	0.978
7.4, 0.15 M salt	4.197	0.992	0.01426	0.985
1.2, 0.15 M salt	0.466	0.982	1.1410	0.996
Water, 0.15 M salt	0.339	0.976	0.4280	0.969

Table 3
Release parameters of loaded diphenhydramine hydrochloride on microspheres and the average AIC values for each kinetic model.

Dissolution medium	Ritger–Peppas model			Peppas–Sahin model, $m = 0.5$			Zero-order model		Higuchi–Fickian model		Hopfenberg model		Optimum model
	$k_1 \times 10^2$	n	AIC	$k_1 \times 10^2$	$k_2 \times 10^2$	AIC	$k_d \times 10^2$	AIC	$k_H \times 10^2$	AIC	K_{Ho}	AIC	
7.4, 0 M salt	0.547	0.67	−88.49	0.860	0.007	−95.15	0.170	−65.91	0.920	−86.51	0.023	−19.37	Peppas–Sahlin
1.2, 0 M salt	0.358	0.77	−76.68	0.500	0.060	−76.28	0.260	−72.29	0.750	−60.26	0.031	−6.52	Ritger–Peppas
Water, 0 M salt	0.196	0.60	−116.76	0.240	0.007	−120.21	0.040	−11.61	0.300	−106.22	0.005	−32.75	Peppas–Sahlin
7.4, 0.15 M salt	1.392	1.21	−60.74	0.940	1.117	−59.37	1.640	−77.12	0.302	−53.42	0.188	−16.07	Zero-order
1.2, 0.15 M salt	1.007	0.50	−95.26	0.980	0.004	−114.53	0.610	−63.69	1.040	−91.17	0.087	−9.28	Peppas–Sahlin
Water, 0.15 M salt	0.637	0.59	−117.50	0.540	0.060	−115.36	0.180	−67.39	0.860	−74.58	0.035	−6.37	Ritger–Peppas

the mobility of DPHAH in the microgels. The results, displayed in Table 2, show that the loaded microparticles exhibit a low drug diffusion coefficient in both the early-time and the late-time approximation equations. The calculated D_E and D_L values are in the range of 4.0×10^{-15}–24.0×10^{-9} cm^2/s, at 37 °C, thus, having the same order of magnitude as those reported in the literature on related systems (Liu, Finn, & Yates, 2005; Siepemann, Elkharraz, Siepmann, & Klose, 2005). The D_E and D_L values indicate that the transport of drug in microparticles is controlled by diffusion phenomena. An increase of the D_E values is observed with the increase of the pH of the release medium and the ionic strength. In pH 7.4 medium D_E values are higher than D_L, this reflecting that, in the initial stage, the rate of drug release from microgels is higher compared with the late stage, meaning that after a certain time the drug release occurs in a controlled and sustained manner and the drug release rates decrease with the release time. When the drug release occurs in pH 1.2 or in water, D_E values are lower than D_L. This indicates that the microgels are in hydrate form at later times and the drug can diffuse more quickly through the increased volume of the hydrogels (Liu, Liu, Liao, & Tian, 2012; Mullarney, Seery, & Weiss, 2006).

3.4. The analysis of the drug transport mechanism

One of the objectives of this contribution was to elucidate the transport mechanism of the drug from the synthesized ionic microgels. In this respect, the data obtained in the drug release experiments were fitted using five different theoretical models and

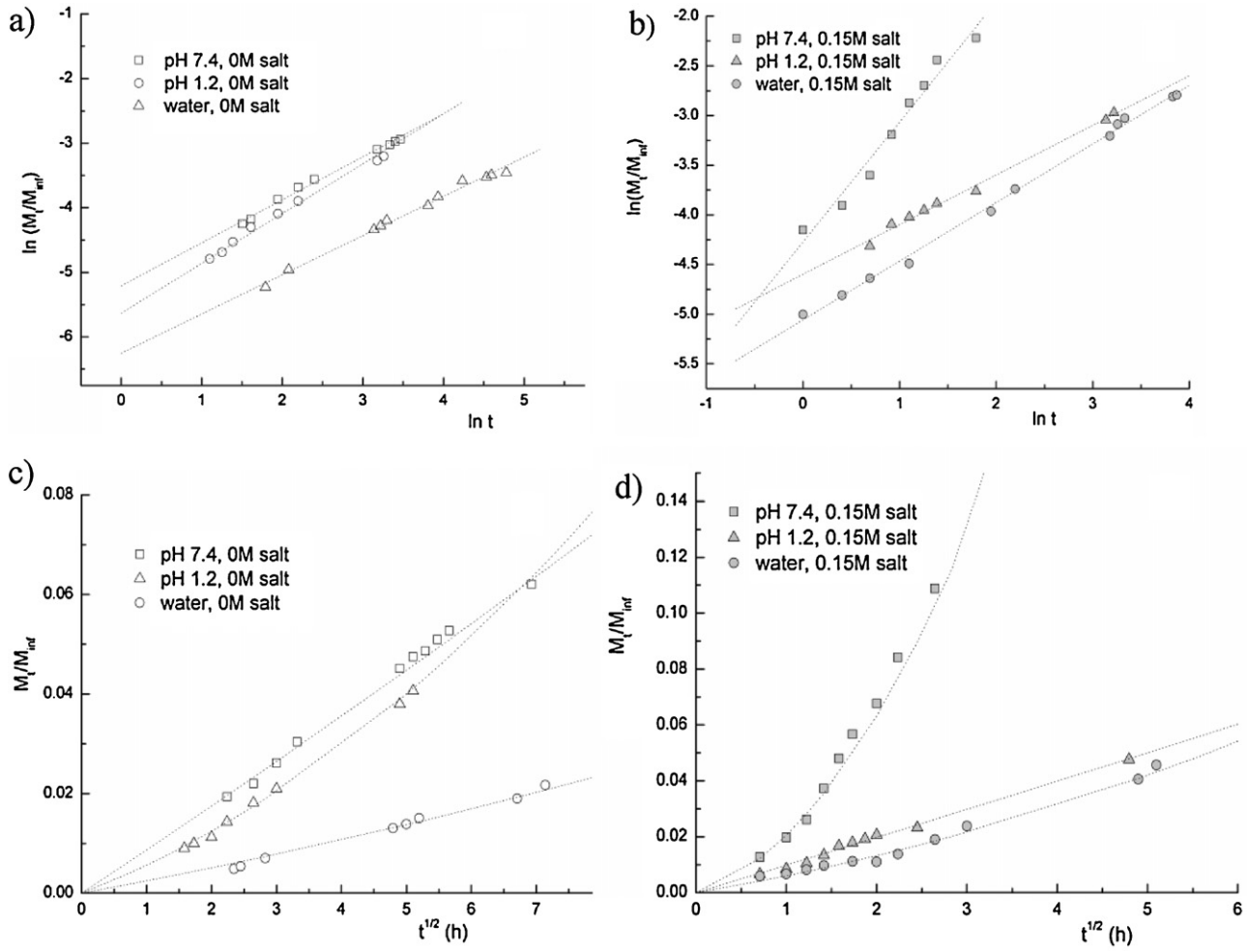


Fig. 4. Release dynamics of diphenehydramine hydrochloride from loaded PCurdMG in different release media at 37 °C: Plot of Ritger–Peppas model (a and b); plot of Peppas–Sahlin model (c and d).

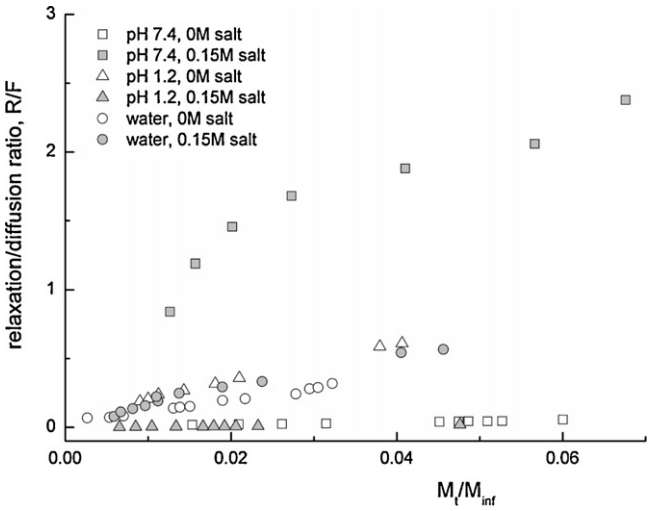


Fig. 5. The R/F ratio as a function of the fractural drug release from PCurdMG.

the calculated kinetic constants are presented in Table 3. These models were based on the effects of the Fickian diffusion and relaxation of the polymer chain during the drug release process. Since the mathematical description of the entire drug release process is rather difficult, owing to the high number of processes (water penetration into the device, matrix swelling, drug diffusion out of the device, electrostatic interactions between drug and charged polymer, moving boundaries) and physical parameters (ionic strength, concentration, porosity, and others), which must be taken into consideration, each model makes certain assumptions. Due to these assumptions, the applicability of the respective models is restricted to certain drug-polymer systems (Serra et al., 2006; Siepmann & Siepmann, 2008). In our systems, the analysis of the Akaike Information Criterion (AIC) values (Table 3) shows that the Peppas–Sahlin (when $m = 0.5$) and Ritger–Peppas models are the ones that statistically describe the experimental data better. Fig. 4 presents the fitting of the experimental plots by those two models. The AIC value of the drug release at pH = 7.4 buffer with added NaCl

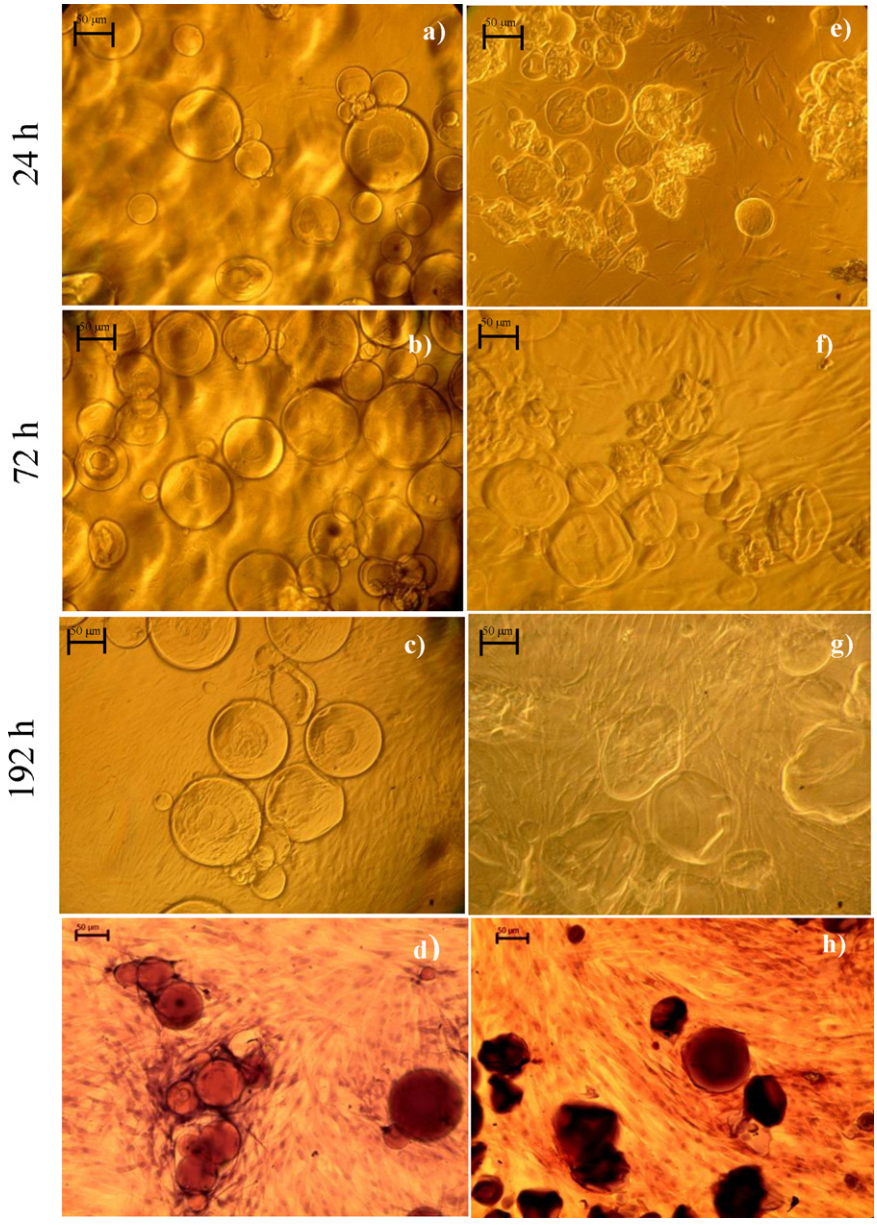


Fig. 6. Live images of RDFs culture in the presence of CurdMG (a, b and c) and PCurdMG (e, f and g) microspheres suspension at different time points. HE staining of RDFs culture after 192 h of co-culturing with CurdMG (d) and PCurdMG microspheres (h).

shows that in this case the experimental data are described by the zero-order model.

Using the Ritger–Peppas model (Eq. (4)), the value of the n exponent, indicative of the drug transport mechanism, was calculated for all the systems (Table 3). The obtained values between 0.500 and 0.772 are an indication that the drug transport mechanism was anomalous. An exception is found at pH 7.4 buffer solution with added NaCl, when $n = 1.209$, indicates a super Case-II transport mechanism. In this case the diffusion is very fast compared with the relaxation process of the polymer matrix.

Using the Peppas–Sahlin equation Eq. (5b) and the parameters estimated in Eq. (6), the ratio between the relaxation contribution R and the diffusional contribution F during the drug release were calculated. The ratio R/F versus the fraction of the drug released from PCurdMG is shown in Fig. 5. It was observed that the ionic strength and the pH of the release medium had an important effect on the DPHAH transport mechanism. At pH = 7.4 with added NaCl, the relaxation of polymeric chain is considerable, leading to a super Case II transport, but in the rest of the cases, the R/F ratio <1 confirms that the release is governed by diffusion.

3.5. Biocompatibility test

The microscopic images of the living cells growing in the presence of CurdMG and PCurdMG microspheres are presented in Fig. 6. PCurdMG microspheres express a higher capacity to seed on the culture plate and closely interact with the cells. After 72 and 192 h of interaction with the cells the PCurdMG are closely surrounded and covered by cell monolayers (Fig. 6f and g). A lower interaction capacity of the non-ionic microspheres (CurdMG) with the culture plate and with the cells, compared with that of the ionic synthesized microspheres (PCurdMG) is observed (Fig. 6b and c).

The microscopic images of the fixed and stained cells in co-culture with CurdMG and PCurdMG materials after a long period of incubation are presented in Fig. 6d and h. A good cell seeding and spreading around both CurdMG and PCurdMG allows to conclude that the synthesized microspheres present a high biocompatibility. The basophil characteristics of PCurdMG and the properties of CurdMG from neutral to slightly acidophil are observed using the HE procedure. The acid/basic properties could explain a better initial attachment to culture substrate followed by a better cell attachment of PCurdMG.

4. Conclusions

The new monobasic curdlan phosphate and curdlan microgels were synthesized by chemical cross-linking with ECH using the w/o inverse emulsion technique. The optical and scanning electron microscopy and FTIR spectroscopy were used to analyze the morphology and the chemical structure of these new microgels. The main characteristics of these new microgels such as the swelling degree in various pH solutions and the exchange capacity were established. The thermal analysis of PCurdMG showed that these microgels present a thermal stability up to 220 °C. The loading and release profiles of DPHAH from PCurdMG were investigated and its diffusion coefficients were calculated. The microgels from non-ionic curdlan were synthesized and characterized, using the same techniques, in order to compare the obtained results for these new anionic microgels. The drug release experiments performed at different pH values and ionic strength of the release medium indicated that the drug release rate increased with the pH and the ionic strength. The mathematical models showed that the drug release mechanism appeared to be anomalous (non-Fickian). On the contrary, at higher pH value and ionic strength (added NaCl) the super-Case II of the drug transport mechanism was observed. Since, the experimental data are not fitted by Hopfenberg model we can conclude that the synthesized microgels are not degradable during the release processes. The uniform distribution of the ionic groups in the microgels structure leads to a retardation of the drug release. A good cell seeding and spreading around the PCurdMG allowed to conclude that the synthesized microgels present a high biocompatibility compared with non-ionic curdlan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.02.014.

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