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Polyelectrolyte complexes of poly[(2-dimethylamino) ethyl methacrylate]/chondroitin sulfate obtained at different pHs: I. Preparation, characterization, cytotoxicity and controlled release of chondroitin sulfate

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ABSTRACT

For the first time, polyelectrolyte complex based on poly[(2-dimethylamino) ethyl methacrylate] (PDMAEMA) and chondroitin sulfate (CS) was prepared. The properties of novel material and precursors were investigated by WAXS, FTIR, TGA, SEM and DLS analysis. The PDMAEMA/CS PECs presented hydrophilic–hydrophobic transition at pHs 6.0, 7.0 and 8.0 whereas the non-complexed PDMAEMA showed such a transition at pH 8.0 and not at pHs 6.0 and 7.0. Studies of CS release from PECs at pHs 6 and 8 confirmed that the samples possess the potential to release the CS in alkaline and not in acidic conditions. Since PECs are thermo-responsive due to the reduction of LCST caused by the increase in pH, the release of CS was dependent on temperature and pH factors. Cytotoxicity assays using healthy VERO cells showed that the complexation between CS and PDMAEMA increased the PECs' biocompatibility related to PDMAEMA. However, the biocompatibility depends on the amount of CS present in the PECs.

1. Introduction

Solution of oppositely charged moieties such as polycation and polyanion results in the formation (or assembly) of polyelectrolyte complexes (PECs) through ionic and H-bond interactions (Follmann et al., 2012). Many factors, including polymer properties (*e.g.*, density of charges and kinds of functional groups), solution properties (*e.g.*, mixing ratio, concentration), external conditions (such as solvent, pH and ionic strength, stirring), *etc.*, may influence the formation, structure and stability of PECs. As well as applications in other fields, PECs are used in a variety of forms in the biomedical field, such as dental adhesives, pulp regeneration, controlled release devices and drug delivery (Follmann et al., 2012; Martins et al., 2011).

Poly[(2-dimethylamino) ethyl methacrylate] (PDMAEMA) has been extensively investigated mainly due to its sensitivity to changes in temperature and/or pH. PDMAEMA presents structural modifications when heated in aqueous media, which results in an LCST-type (lower critical temperature solution) phase diagram. In this transition, the hydrophilic structure of PDMAEMA changes to a hydrophobic structure as it is heated above the LCST (Dong et al., 2012). In such a condition, the interactions among PDMAEMA chains and water molecules are disrupted and polymer–polymer interactions prevail and the polymer collapses (Dong et al., 2012). The LCST of PDMAEMA occurs in the range of 32–53 °C (Zhang et al., 2006). For PDMAEMA, the value of LCST is related to the average molecular weight and to the pH of the medium (Mao et al., 2011). PDMAEMA is a weak polyelectrolyte, whose charge density can be altered by varying the pH, because in a given pH condition the tertiary amine groups may (or may not) be protonated. The pK_a of amine groups of PDMAEMA is around 7.0 (Dong et al., 2012). Therefore at a pH < 7 this polymer presents the tertiary amine groups in the ionized form and, in this

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condition, the PDMAEMA acts as polycation (Dong et al., 2012; Zhang et al., 2006). So, this polymer can interact with polyanionic polymers, enzymes and DNA through electrostatic interactions and their responses to pH and temperature are useful in the design of new controlled-release drug systems (Loh et al., 2013; Wei et al., 2011; Zhang et al., 2006).

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and *N*-acetyl galactosamine, sulfated at both/either the C4 and/or C6 positions (Cho et al., 2014). CS is an important structural component in connective tissues and cartilage. CS is extracted mainly from bovine aorta, and is a water-soluble biopolymer that has been widely used to treat arthritis-related diseases (Piai et al., 2009).

It is an important component in protein complexes found in the extracellular matrix of conjunctive animal tissues. CS forms networks with collagen allowing the CS to be responsible for the good mechanical properties of such connection tissues (Fajardo et al., 2010). On the other hand, the high water solubility of CS limits the application of this biopolymer in medical and pharmaceutical formulations (Cavalcanti et al., 2005; Cho et al., 2014).

Due to its cationic nature and high charge density in acidic environments, PDMAEMA can form insoluble-water complexes with anionic moieties such as CS. In this work, the investigated hypothesis is that PECs based on PDMAEMA/CS can be an alternative to keep CS in the PEC matrix, reducing the biopolymer solubility but, at the same time, maintaining its bioavailability through sustainable release of CS in specific conditions (Fajardo et al., 2010).

This work aims to develop new polymeric materials based on PECs made through complexing PDMAEMA and CS in different pH conditions and to evaluate the release profile of CS from PDMAEMA/CS PECs. Some physical–chemical properties of as-prepared materials were investigated and are discussed in this paper, such as changes in the effectiveness of unlike polymer interactions as the conditions (pH of PEC-forming solution and PDMAEMA/CS ratio) are altered. Moreover, the influences of pH and temperature on the CS release profile and also on PEC thermal stability, crystallinity, cytotoxicity and morphology were evaluated. These targets are justified by the excellent properties of PDMAEMA and CS. It is expected that PDMAEMA/CS PECs may have opportunities for further potential uses in the biomaterial field.

2. Experimental

2.1. Materials

Chondroitin sulfate (Mv = 22,000 g/mol) was kindly supplied by Solabia (Maringá, Brazil). 2-(Dimethylamino) ethyl methacrylate (DMAEMA); 2,2-azo-Bis-isobutyronitrile (AIBN); glycine and 1,9-dimethyl-methylene blue (DMB) were purchased from Sigma–Aldrich. Other reactants such as hexane and tetrahydrofuran were also utilized in this work. All reactants were used as received without further purification.

VERO (African green monkey kidney) cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco®, Grand Island, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco®) and 50 μg of ml^{-1} gentamycin, in an incubator set at 37 °C, 5% CO_2 and 95% relative humidity. The cells were expanded when the monolayer reached confluence at day 3. After reaching 80% confluence, cells were digested by using a trypsin/EDTA solution (0.25% trypsin–Gibco®, and 1 mmol l^{-1} EDTA).

2.2. Methods

2.2.1. Synthesis of poly[(2-dimethylamino) ethyl methacrylate] (PDMAEMA)

The methodology employed for the preparation of PDMAEMA is based on the method published by You et al. (2007) with some modifications. The methodology used is described in detail in the Supplementary material (SM).

2.2.2. Preparation of polyelectrolyte complexes (PECs of PDMAEMA/CS)

The following procedure was adopted: aqueous solutions of PDMAEMA (certain amounts in 80 ml of distilled water) and also of CS were prepared at pHs 1, 3 and 5. The final concentration of CS aqueous solution was 25% (w/v). Afterwards, the previously prepared PDMAEMA solutions, at the desired pH (1, 3 and 5), were slowly dropped into CS solutions 25% (w/v) of the same pH, at room temperature and under magnetic stirring. The resultant mixture was allowed to stand for a further 24 h. Afterwards an aliquot of 400 μl was collected from the supernatant for CS quantification as described in the next section. Then, the obtained precipitate was isolated by centrifugation (30 min) at 2 °C. During the purification process, NaOH 0.2 mol l^{-1} aqueous solution was added to the system and the pH adjusted to 7. Finally, the PECs of PDMAEMA/CS were frozen and lyophilized at −55 °C for 24 h.

In order to evaluate the effect of the PDMAEMA/CS ratio and pH factors in the formation of PECs, a 2^2 factorial design with a central point was performed and the software Design-Expert®, version 7.0, was used for statistical analysis. In this factorial design, the PDMAEMA solution concentration % (w/v) and the pH of previously prepared PDMAEMA and CS solutions were varied in three levels, as summarized in Table 1. The factorial design was performed in triplicate at the central point and in duplicate in other four conditions.

To predict the best conditions for obtaining the PECs, a second-order polynomial equation

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_{ij} \quad (1)$$

was applied for correlating the response (Y) to factor levels, with Y being the yield (in terms of per cent of CS mass incorporated in the PEC matrix), and β_0 the average value considering all the experiments. β_i and β_{ii} are, respectively, the first- and second-order coefficients associated with each independent factor i,β_{ij} are the coefficients of the interaction factors, and x_i is the coded value of the factor i. From the quadratic equation (Eq. (1)), a response surface plot was generated. An analysis of variance (ANOVA) to assess the statistical significance of effects associated with the studied response factors was conducted.

2.2.3. Quantification of chondroitin sulfate (CS) complexed with PDMAEMA

For each test, 400 μl of supernatant (remaining after precipitating the PDMAEMA/CS PECs) were mixed with 5.0 ml of 1,9-dimethyl-methylene blue (DMB) solution (Kafienah and Sims, 2014). The DMB solution was prepared by solubilization of 16.0 mg in 1.0 l of solution containing glycine (40 mmol l^{-1}), NaCl (40 mmol l^{-1}) and 95 ml of HCl 0.10 mol l^{-1}. So, each collected aliquot was

Table 1
Factors and levels used in the factorial planning 2^2.

Factor	Unit	Level (−1)	Level (0)	Level (+1)
PDMAEMA	% (w/v)	1.9	2.2	2.5
pH	–	1	3	5

directly introduced into 5.0 ml of DMB aqueous solution at pH 3.0. The absorbance measurements of the 1,9-dimethyl-methylene blue/chondroitin sulfate (DMB/CS) metachromatic complex were taken immediately after mixing and homogenizing the samples. An analytical curve correlating the absorption of DMB/CS complex to the concentration of CS, from aqueous solutions of CS ($1.0\,mg\,l^{-1}$–$50\,mg\,l^{-1}$) was built ($R^2 = 0.9962$) to quantify the amount of non-complexed CS with the PDMAEMA. The absorbance of DMB/CS complex was measured on UV–vis spectrophotometer (Femto, model 800XI, Brazil) at $\lambda = 525$ nm (Kafienah and Sims, 2014). The encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulationefficiency} = \frac{[\text{CSi}] - [\text{CSs}]}{[\text{CSi}]} \times 100\% \quad (2)$$

where [CSi] is the amount of CS in the solution before mixing and [CSs] is the amount of CS remaining in the supernatant.

2.3. Polyelectrolyte complex characterization

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were recorded using a Fourier transform infrared spectrophotometer (Shimadzu Scientific Instruments, Model 8300, Japan), operating in the 4000–500 cm^{-1} range, at a resolution of 4 cm^{-1}. FTIR spectra were obtained from samples of KBr pellets at 1% concentration.

2.3.2. Thermogravimetric analysis (TGA)

TGA was performed from 25 to 700 °C at a heating rate of 10 °C min^{-1} under $N_{2(g)}$ flowing at 50 ml min^{-1} in a TGA-50 Thermogravimetry Analyser (Netzch, model STA 409 PG/4/G Luxx, USA).

2.3.3. Wide-angle X-ray scattering (WAXS)

The WAXS profiles were recorded on a diffractometer (Shimadzu Scientific Instruments, model XRD-600, Japan) equipped with Ni-filtered Cu-Kα radiation. The WAXS profiles were collected in the scattering range $2\theta = 5$–70°, with a resolution of 0.02°, at a scanning speed of 0.1° min^{-1}. The analyses were performed by applying an accelerating voltage of 40 kV and a current intensity of 30 mA.

2.3.4. Scanning electron microscopy (SEM)

Immediately after obtaining the samples, they were frozen in liquid nitrogen, lyophilized for 48 h at −55 °C and fractured. The PEC morphologies were investigated through images from a scanning electron microscope (SEM, Shimadzu Scientific

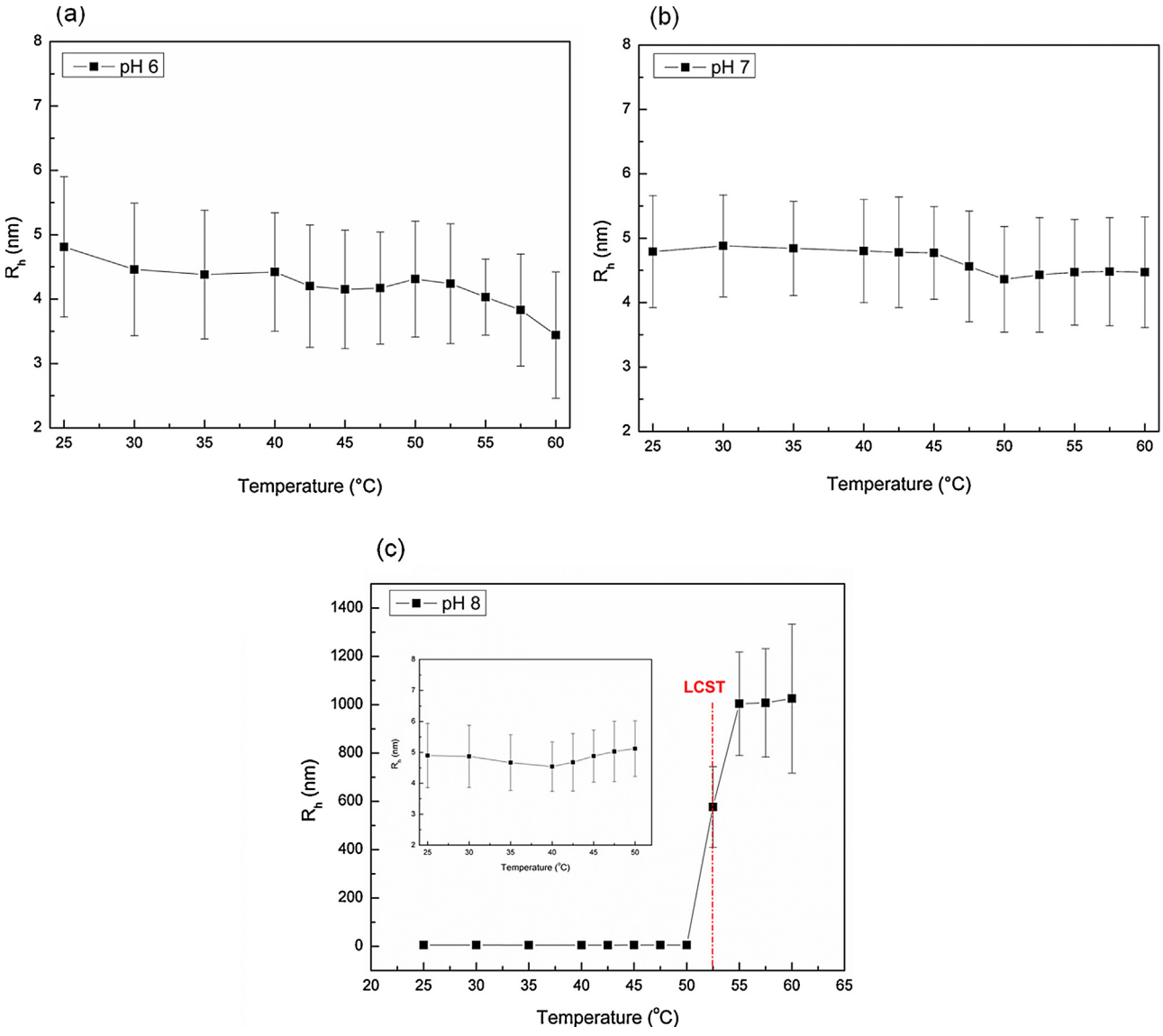


Fig. 1. Effect of temperature on hydrodynamic radius (R_h) of the PDMAEMA solutions at (a) pH 6.0, (b) pH 7.0 and (c) pH 8.0.

Instruments, model SS 550, Japan). PEC surfaces were sputter-coated with a thin layer of gold before SEM visualization. The SEM images were taken by applying an electron accelerating voltage of 10–12 kV.

2.3.5. Measurements of dynamic light scattering (DLS)

The hydrodynamic radius (R_h) was determined using a Malvern apparatus (Zetasizer Nano ZS, UK) with an He–Ne laser (633 nm) at a fixed angle of 173°. Buffer solutions with an ionic strength of 0.10 mol l^{-1} at pHs 6.0, 7.0, 8.0 and 9.0 were used. (USP30–NF25, 2014) The LCST was determined through R_h curves as a function of temperature. To evaluate the LCST of linear and of complexed PDMAEMA, a PDMAEMA solution (20 mg ml^{-1}) and PDMAEMA/CS PECs solution of 0.20 mg ml^{-1} were employed, respectively. All measurements were performed, in triplicate, after the sample achieved thermal equilibrium at initial temperature analysis (ca. 25 °C).

2.3.6. In vitro chondroitin sulfate release

The *in vitro* assays for evaluating CS release from PECs were performed in two different environments: buffer solutions at pHs 6.0 and 8.0. In a dissolution apparatus (Ethik Technology, model 299-6TS, Brazil), 350 mg of PECs were deposited in a sealed flask with 150 ml of buffer solution under constant stirring (60 rpm), at 36.5 or 60 °C. At a desired time interval, an aliquot (400 μl of supernatant containing the PECs) was removed from the flask and directly added to 5.0 ml of DMB buffer solution, to quantify the released amount of drug through UV measures, as described previously. All the experiments were performed in duplicate and the fraction of CS released was calculated from the following equation:

$$\text{Fraction of CS released} = \frac{\text{amountofCSrelesead}}{\text{amountofCSencapsulated}} \times 100\% \quad (3)$$

2.4. Cytotoxicity assays

The cytotoxicity activities of samples against healthy VERO cells were determined by sulforhodamine B assay, as described by Skehan et al., (1990). The cells were seeded in 96-well tissue plates (TPP–Techno Plastic Products, Trasadingen, Switzerland) at a density of 2.5×10^5 cells in 100 μl of medium for 24 h in the CO_2 incubator. The samples (PEC or PEC precursors) were dissolved in water and they were added to the medium at various concentrations. After incubation for 48 h, the cell monolayers were washed using 100 μl of sterile phosphate buffered saline (PBS), fixed with trichloroacetic acid and stained for 30 min with 0.4%-w/v sulforhodamine B (SRB–Sigma Chemical Co., USA) dissolved in 1%-v/v acetic acid. The dye was removed by four washes with 1%-v/v acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [Tris (hydroxymethyl) aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader (Power Wave XS, BIO-TEK®, USA).

3. Results and discussion

3.1. Hydrophilic/hydrophobic transition of PDMAEMA

In order to assess the LCST of PDMAEMA at different pH and temperatures, aqueous solutions of this polymer were subjected to analysis through dynamic light scattering (DLS). The hydrodynamic radius (R_h) of the sample was measured in aqueous solution at pHs 6.0, 7.0 and 8.0 at temperatures from 25 to 60 °C (Fig. 1). It was not possible to carry out measures at pH 9.0 due to aggregation of PDMAEMA, even at room temperature. At pHs 6.0 and 7.0 a decreasing trend in the R_h was observed as the temperature was increased, but no aggregation was observed since these pH conditions are below or close to the pK_a value of PDMAEMA (Yancheva et al., 2007). So, at pHs 6.0 and 7.0 the PDMAEMA presented only a very slight decrease in R_h (bearing in mind the measure errors) as the temperature was changed from 25 to 60 °C (Fig. 1). This indicates a decrease in the affinity of the polymer to the solvent, but such a decrease is not enough to induce the collapse of polymer coils. At pH < 7.0 the tertiary amine groups present along the PDMAEMA chains are protonated, a fact that favors its solubility in water and prevents the aggregation process. The data show that the decrease in R_h at pH 6.0 occurs at ca. 55 °C; and at pH 7.0 at ca. 45 °C. On the other hand, at pH 8.0 a slight decrease in R_h was observed at 35–42 °C and after a sudden increment in the hydrodynamic radius (ΔR_h = 998.7 nm) occurred at 52 °C. The slight decrease in R_h was associated with a gradual lowering in the affinity of polymer coils with solvent and the strong increment was attributed to the collapse of polymer coils leading to aggregation. Therefore, at pH 8.0 the PDMAEMA presents enough hydrophobic characteristic to cause the occurrence of LCST at 52 °C. The total (at pH 6.0) or partial (at pH 7.0) ionization of amine groups in PDMAEMA chains prevents the occurrence of collapse of polymer coils even when the temperature is increased up to 60 °C.

3.2. PEC preparation

The 2^2 factorial design was utilized to study the best pH and PDMAEMA concentration % (w/v) conditions for the formation of PECs (see Table 1). The PEC formation was observed in all studied levels of pH and PDMAEMA concentration and the values of the yield (in terms of per cent of CS mass incorporated on the PEC matrix) are summarized in Table 2. Fig. 2 shows photos of each PEC formulation described in Table 2.

The ANOVA obtained after the application of Eq. (1) to the data collected on the 2^2 factorial design is illustrated in Table 3.

Considering the two studied factors, only the pH significantly influenced the yield of CS mass incorporated on PEC matrices, since the p value was lower ($p < 0.05$) (see Table 2). On the other hand, the effect of the concentration of PDMAEMA and the interaction effect between this factor and the pH used for PEC formation showed $p > 0.05$, and thus they did not significantly affect the complexation process that occurred between PDMAEMA and CS. Associating these data with the response surface (Fig. 3), obtained by applying the quadratic model (Eq. (1)), it can be anticipated that higher yields of CS mass incorporated on PECs occurred when the matrices were prepared at pH 1.0 (level-1). The decrease in pH favors the protonation of the tertiary amine groups present in the PDMAEMA chains, allowing more effective interaction between PDMAEMA and CS.

Table 2
Yield of CS-complexed with PDMAEMA in each assay for factor 2^2.

Sample	Factor		Response	
	PDMAEMA	pH	Yield (%)	PDMAEMA/SC mass ratio
PEC1	(−)	(−)	98.2 ± 0.8	24/76
PEC2	(+)	(−)	97.9 ± 0.6	29/71
PEC3	(−)	(+)	93.8 ± 0.4	24/76
PEC4	(+)	(+)	91.8 ± 2	30/70
PEC5	(0)	(0)	94.4 ± 2	30/70

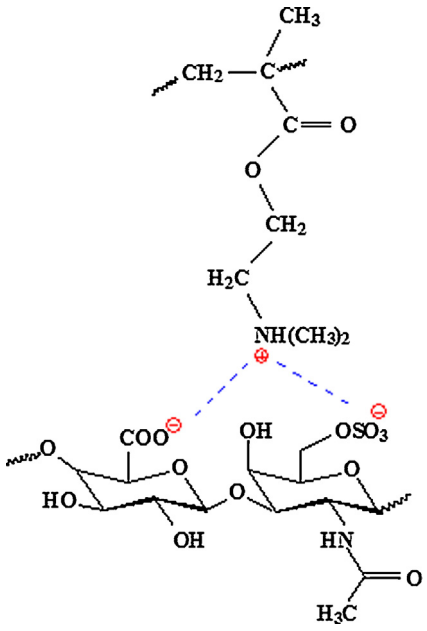
Fig. 2. Photographs of PECs obtained after lyophilization process.

PEC1 and PEC2 samples were selected for further study due to the higher yield of CS mass incorporation in these PEC matrices. The complexation process of PDMAEMA and CS is represented in Scheme 1.

3.3. Characterization of PECs through Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of PDMAEMA, CS, PEC1 and PEC2 are shown in Fig. 4. A well-defined band at 1730 cm^{-1}, attributed to the stretching of C=O groups of esters, was observed on the PDMAEMA FTIR spectrum. Other characteristic bands appear at 2772, 2836 and 1154 cm^{-1} on the PDMAEMA FTIR spectrum, attributed to C—H and C—N stretching, respectively. (Mao et al., 2011; Patel et al., 2012; Zhang et al., 2011) The bands related to the vibration of C—H bonds existing in $-N(CH_3)_2$ groups of PDMAEMA chains occur, but at less intensity on the PEC2 FTIR spectrum than on the PEC1 FTIR spectrum. This was due to the smaller amount of PDMAEMA in PEC2 compared to CS. Furthermore, these results indicate the good experimental conditions used for obtaining the PEC1, compared to the conditions for obtaining the other PECs.

The bands at 1644 and 1429 cm^{-1} on the CS FTIR spectrum possess considerable intensity and were assigned to the asymmetric and symmetric axial deformations of carboxylate anion. On the other hand, these bands presented lower intensity on the PEC1 and PEC2 FTIR spectra than on the CS spectrum, a fact that confirms the complexation between $-COO^-$ groups of CS and amine groups of PDMAEMA (Scheme 1). The bands at 1240 and



Scheme 1.

Table 3
ANOVA results of factorial design 2^2.

Source	Squares sum	Freedom degrees	Squares mean	*F* value	*p* value
Model	61.36	4	15.34	6.47	0.0229
PDMAEMA	2.64	1	2.64	1.12	0.3315
pH	55.12	1	55.12	23.25	0.0029
PDMAEMA x pH	1.45	1	1.45	0.61	0.4647
$PDMAEMA^2$	2.15	1	2.15	0.90	0.3782
Pure error	14.23	6	2.37	–	–
Total	75.59	10	–	–	–

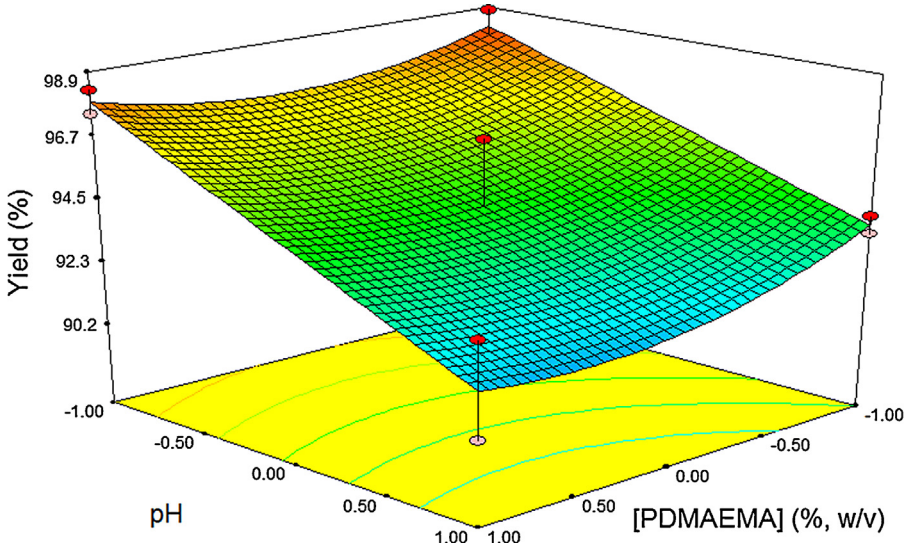


Fig. 3. Response surface obtained from the quadratic model (Eq. (1)). The equation obtained from factorial planning 2^2 and, that represents the quadratic model was: $y = 94.43 - 0.57*A - 2.62*B - 0,43*A*B + 0.99*A^2$.

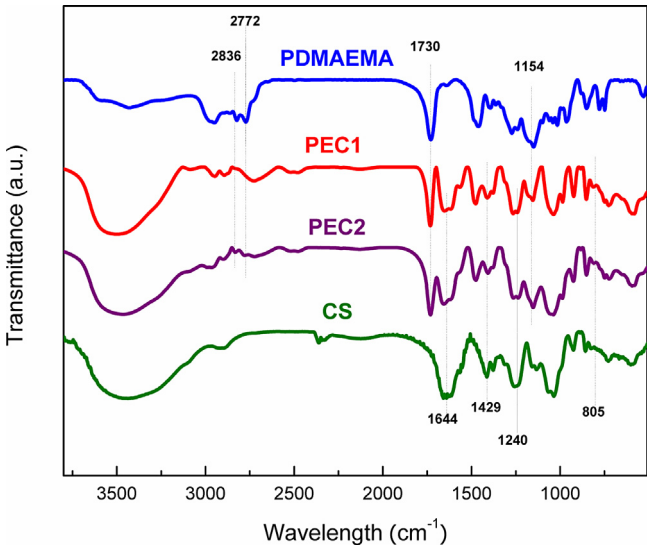


Fig. 4. FTIR spectra of PDMAEMA, CS, PEC1 and PEC2.

806 cm^{-1} were assigned to the axial asymmetric deformation of S=O bonds and to the stretching of C—O—S groups, respectively (Grant et al., 1987). It was observed that these bands occur with similar intensities on CS and PEC FTIR spectra. This was due to the higher amount of CS present in PECs and the fact that pK_a of $-OSO_3^-$ groups belonging to CS is close to 2.0. So, at pH 1.0 these groups interact electrostatically with the positively charged tertiary amine groups present in the structure of PDMAEMA.

3.4. Characterization of PECs through thermogravimetric analysis (TGA)

TGA curves of different PECs and precursors (CS and PDMAEMA) are shown in Fig. 5. Two stages of mass loss were observed in the TGA curves, the first one occurring in the 25–125 °C range being attributed to water evaporation. It should be noted that those samples showed different water content. CS presented higher water content than PDMAEMA, since CS possesses carboxylate ($-COO^-$) and sulfonate ($-OSO_3^-$) groups which are highly hydrophilic and could strongly interact with water molecules through dipole–dipole or ion–dipole forces. Therefore, the amount of CS incorporated influences the water content in the samples. For its part, PDMAEMA possesses tertiary amine groups that could provide some dipole–dipole interactions with water molecules. However, PDMAEMA is more hydrophobic than CS. Interestingly, the PECs presented higher amounts of water than their precursors. During the complexation, water molecules were probably entrapped in the PEC matrix, contributing to the increase in the amount of water compared to the precursors.

The degradations of PDMAEMA, CS, PEC1 and PEC2 occur in the second stage of weight loss and are clearly shown in the TGA curves in Fig. 5. PDMAEMA degradation starts at 230 °C and ends at about 450 °C. CS showed a high weight loss in the range of 230–250 °C, however this polymer showed higher thermal stability than PDMAEMA, probably due to the formation of sulfated residues. The same behavior evolving CS and CS derivatives was observed in 2003 by Wang et al., (2003) and more recently in a work by our research group where heparin was incorporated into fibroin nanofibres. (Cestari et al., 2014) Like CS, heparin possesses sulfate groups that become sulfated residues above 500 °C. Up to 280 °C the TGA curves for PECs are more similar to the TGA curve of CS than that of PDMAEMA. However, above this temperature the TGA for PECs became very dissimilar to the CS and also to the PDMAEMA TGA curves and only small residues (less than 5%) were observed at 700 °C. This is an unexpected result. To better understand the effect, a sample was prepared just by mixing the PDMAEMA and CS (in powder, so it is not a PEC) with a ratio of 1/1 (w/w) and analysed through TGA (see Fig. S1, Supplementary material). It can be seen that the TGA curve for this sample is very similar to the CS TGA curve. In the TGA curve of 1/1 PDMAEMA/CS the residue amount remained at 700 °C and almost half the amount of residue remained at 700 °C in the TGA curve for neat CS. From these results it can be inferred that the interactions existing between CS and PDMAEMA on PEC1 and on PEC2 should be responsible for the degradation profile of PECs observed in Fig. 5. Probably, electrostatic interactions among groups of opposite charges present in PDMAEMA and CS chains are responsible for the higher thermal stability of PEC1 and PEC2 than of PDMAEMA while allowing almost complete degradation of PECs as compared to the CS TGA curve at 700 °C.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2014.10.017.

3.5. Characterization of PECs through WAXS

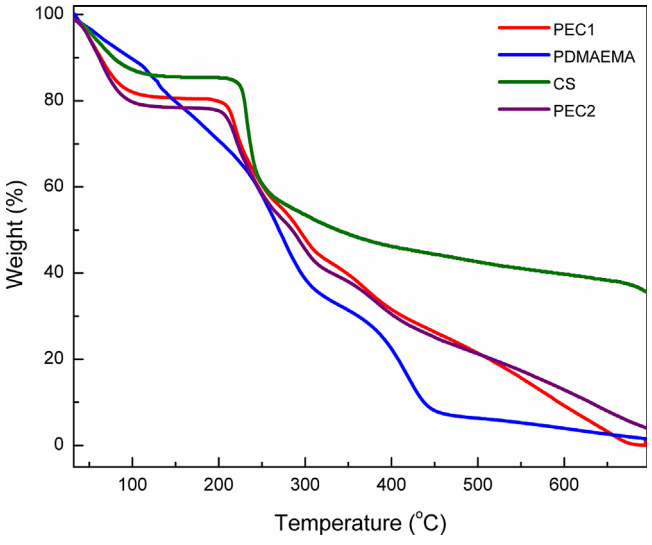


Fig. 5. TGA curves of PDMAEMA, CS, PEC1 and PCE2.

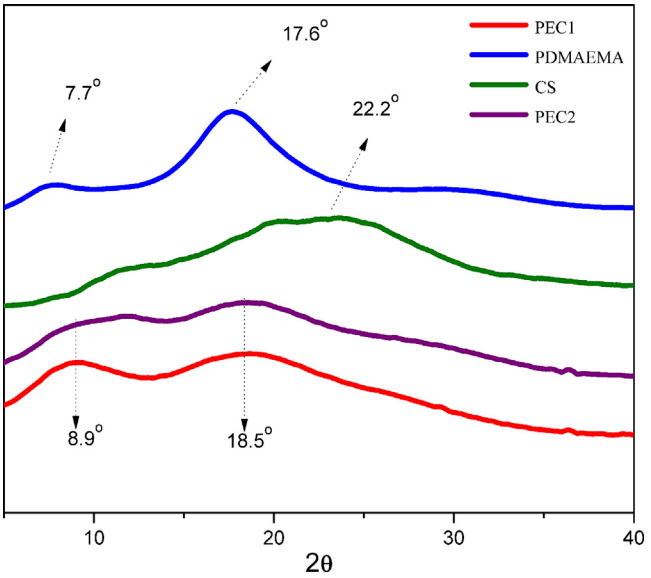


Fig. 6. WAXS profiles of PDMAEMA, CS, PEC1 and PEC2.

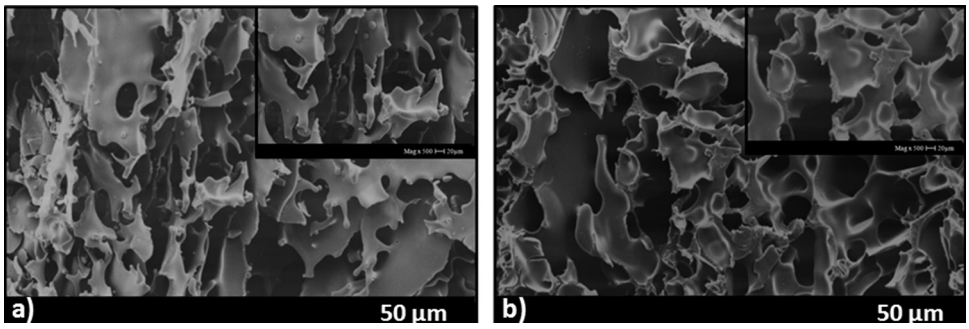


Fig. 7. SEM images of PEC1 (a) and PEC2 (b) obtained after lyophilization process.

Fig. 6 shows the WAXS profiles of CS, PDMAEMA, PEC1 and PEC2. The WAXS profile of CS exhibited a broad diffraction peak with low intensity that occurs in the range from 10° to 30° (centred at $2\theta = 22.2°$) and characterizes its low degree of crystallinity. The WAXS profile of PDMAEMA exhibited two broad and low diffraction peaks at $2\theta = 7.7°$ and 17.6°, which confirm the presence of slightly ordered regions on the PDMAEMA structure, which are formed by dipole–dipole bonds among the tertiary amine and other groups. On the other hand, significant differences in the WAXS profiles of precursors (PDMAEMA and CS) and PECs can be observed. From the WAXS profiles of PECs shown in Fig. 6, it can be seen that PEC1 and PEC2 are amorphous, a fact proved by the occurrence of two broad diffraction peaks of very low intensity at $2\theta = 8.9°$ and 18.5°.

3.6. Characterization of PECs by scanning electron microscopy (SEM)

SEM images of PEC1 and PEC2 obtained after the lyophilization process are shown in Fig. 7. The micrographs show that these materials presented pores, with the morphology of PEC2 indicating a slightly more compacted material than that of PEC1. The high porosity and compaction of PEC2 is probably due to the highest proportion of PDMAEMA being present in the PEC2 matrix (Wei et al., 2011). The analysis of the magnified image inserted into the PEC1 SEM micrograph showed the existence of small fragments in the PEC1 matrix, a fact that demonstrates the greater fragility of PEC1 compared to PEC2. The fragments present in PEC1 morphology probably occur as a result of the higher amount of CS in this sample.

3.7. Characterization of PECs through dynamic light scattering (DLS)

The sensitivity of PECs to changes in temperature and pH were investigated through DLS measurements of aqueous dispersions at pHs 6.0, 7.0 and 8.0, and in a temperature range of 23–60 °C (see Fig. 8).

The increase in temperature caused a significant reduction in the hydrodynamic radius (R_h) of particles in all the investigated formulations. In contrast to what was observed for the linear (not complexed) PDMAEMA, the R_h *vs* temperature curves for PEC1 and PEC2 presented a significant reduction in the three studied pHs, but no abrupt increase in the R_h *vs* temperature curve was observed in any of the cases. In this case, the lower mobility of PDMAEMA due to the 3D matrix formation avoided the chain collapsing or coil aggregation but the R_h of the PDMAEMA chains decreased much more than that of linear PDMAEMA. The loss of entropy causes an increment in the free energy of mixing compared to linear PDMAEMA. So the change in T causes a much bigger decrease in the affinity of this polymer to water molecules (in this case, 3D swollen PEC). PEC1 and PEC2 even presented significant reductions in R_h as the temperature was increased, with the reductions for PEC1 at pH 6.0, 7.0 and 8.0 (Fig. 8a–c, respectively) being more intense than for PEC2 at the respective pHs. This shows that the PDMAEMA/CS ratio influences the amount of R_h reduction of PDMAEMA within the PECs.

The transition temperature (LCST) of each sample in a fixed pH condition was determined from the R_h *vs* T plot as presented in Fig. 8. The error associated with each determination is ±2 °C. The values of LCST for PDMAEMA and for the PECs are presented in Table 4. The results revealed that the LCST exhibited strong dependence on the pH condition used for the measure, whereas the increase in pH decreased the value of LCST for the PECs (Table 4), as expected.

The dependence of LCST on the pH was attributed to the ionization of $-N(CH_3)_2$ groups present in PDMAEMA chain segments. In a condition of $pH < pK_a$ ($pH < 7$), such groups are ionized and the complexation with the groups of CS is favoured, and in this circumstance, the energy required for promoting the phase transition in the PDMAEMA/CS system became higher. This explains the higher value of LCST at pHs 6.0 and 7.0. So, at $pH > 7$ some of the $-N(CH_3)_2$ groups are not ionized and the absence of electrostatic interactions causes destabilization of PECs allowing a considerable decrease in LCST (Table 4).

Furthermore, the increase in pH favors intra- and inter-chain interaction of PDMAEMA molecules due to the hydrophobic character acquired by this polymer. It should be noted that at pH 9.0, aggregation of PDMAEMA occurred (results not shown), a fact that prevented the LCST measurement of PDMAEMA at this pH. Fig. S2 (Supplementary material) illustrates the possible PDMAEMA structural changes that occur in different pH and T conditions. The higher the pH in which the PEC is dispersed, the lower is the LCST. But the PDMAEMA/CS ratio also has an influence since PEC2 showed higher LCST values than PEC1 (see Tables 1 and 4).

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At pH 6.0 and 7.0 the PECs exhibited a sharp change in R_h *vs* T (associated with hydrophilic/hydrophobic transition), while the non-complexed PDMAEMA did not. On the other hand, at pH 8.0 the interactions among CS-complexed PDMAEMA chains with water are weakened, favouring the occurrence of LCST at lower temperatures than for neat PDMAEMA (Table 4). As mentioned earlier, this is due to the loss of entropy (related to lower mobility) of PDMAEMA chains once physically cross-linked on 3D matrices. This result is of utmost importance and may be useful in the development of new biomaterials based on PDMAEMA/CS with a temperature response property (LCST) near to body temperature.

3.8. In vitro release of chondroitin sulfate (CS)

In vitro CS release assays were performed using only PEC2 as a sample. The assays were conducted under pHs 6.0 and 8.0 (below

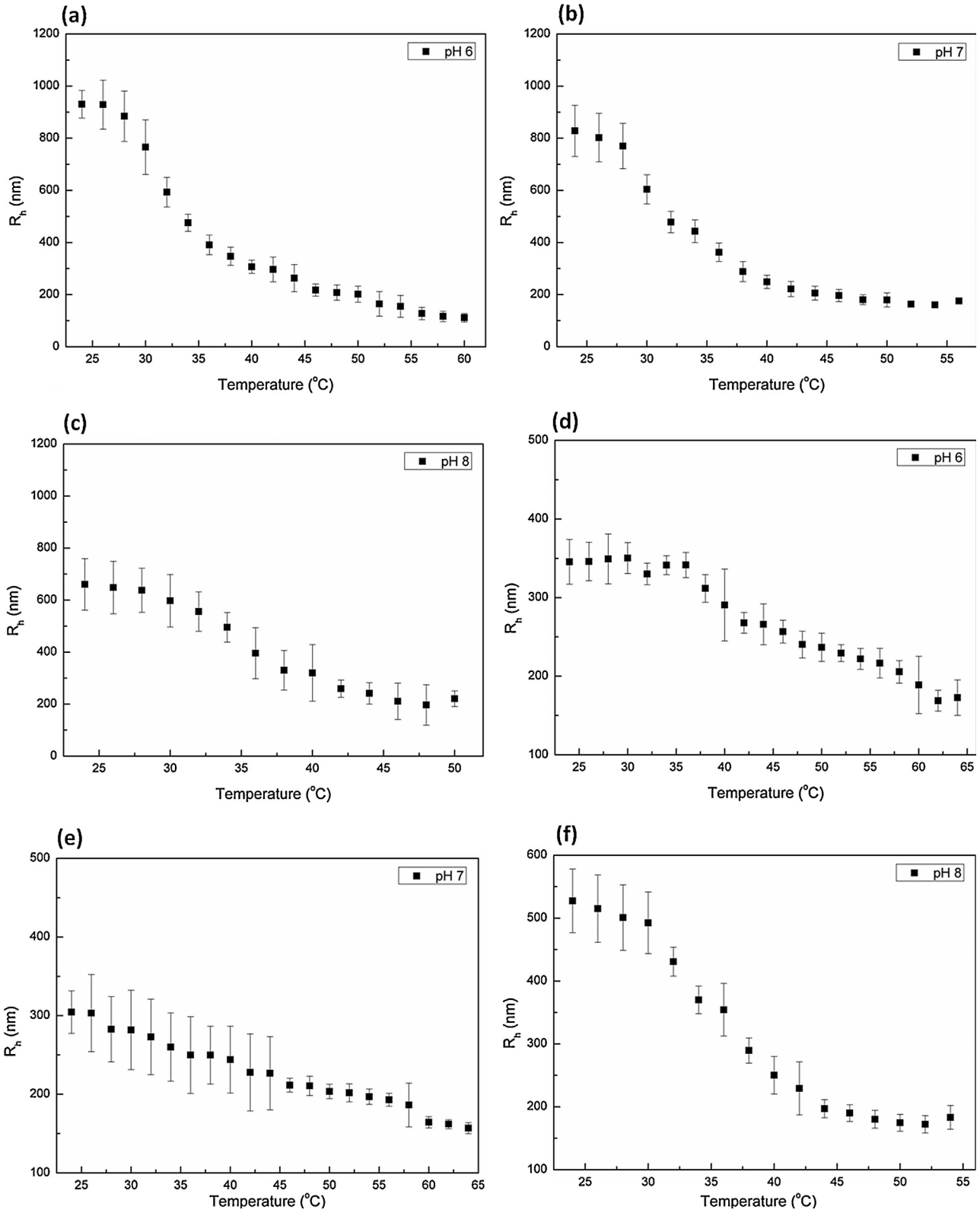

Fig. 8. Dependences of hydrodynamic radius (R_h) with temperature at pH 6.0, 7.0 and 8.0: PEC1 (Fig. 8a–c) and PEC2 (Fig. 8d–f).

and above the pK_a of PDMAEMA), at 36.5 °C (below the LCST condition) and at 60 °C (above the LCST condition). Fig. 9 shows the profiles of CS release obtained in these temperature and pH conditions.

The release of CS was suppressed at pH 6.0 but favoured at pH 8.0, since ca. 1.5% of CS incorporated into PEC2 hydrogel was released at pH 8.0 (Fig. 9a and b) until equilibrium was reached. This result is in line with the data obtained from DLS

Table 4
LCST values of PEC1, PEC2 and PDMAEMA obtained at different pH-conditions.

	LCST (°C)		
pH	PEC1	PEC2	PDMAEMA
6	58 ± 2	62 ± 2	–
7	52 ± 2	60 ± 2	–
8	46 ± 2	50 ± 2	52 ± 2

measurements. At pH 8.0 the electrostatic interactions existing between PDMAEMA and CS are broken due to deprotonation of tertiary amine groups. Thus, the release of CS is favored. On the other hand, at pH 6.0 the electrostatic interactions between PDMAEMA and CS are effective and the CS release is suppressed. Therefore, the CS remains complexed with the PDMAEMA at pH conditions below the pK_a of tertiary amine groups and only a very small amount of CS is released.

The temperature also significantly influenced the profile of release curves as can be seen in Fig. 9. At pH 8 the equilibrium for CS releasing occurred ca. 5.5 times more rapidly at 60 °C than at 36.5 °C. So, below the LCST (36.5 °C), the PDMAEMA chains are fully extended (Fig. S2) and the complexation stabilizes the CS in the PEC matrix. However, when the temperature is raised to 60 °C (above the LCST), the R_h of PDMAEMA chains decreases due to the increased hydrophobicity (Li et al., 2013), the interactions between PDMAEMA and CS are weakened and the CS is released faster and equilibrium is reached in around 3 h. On the other hand, at 36.5 °C the release equilibrium was achieved in ca. 12.5 h.

So pH and temperature are factors that can be used for tuning the rate of CS release from PEC based on PDMAEMA and CS.

3.9. Evaluation of mechanism for CS release from the PDMAEMA/CS matrix

The release can occur as a diffusional transport process and/or as a partition phenomenon in which the partitioning of solute(s) between the solvent phase and the hydrogel phase takes place. Ritger and Peppas (1987) proposed a well-known semi-empirical model that describes the mechanism related to the transport of solutes from a flexible matrix that is given by the equation

Table 5
Values of diffusional exponent (n) and constant (k) obtained by application of Ritger–Peppas[22] model (Eq. (4)) on release curves of CS from PEC2 (Fig. 9) in mediums at pH 8.0 and different temperatures.

Temperature (°C)	N	k (10^{-3})	R^2
36.5	0.93	1.43	0.99
60.0	0.93	5.37	0.96

$$\frac{M_t}{M_\infty} kt^n \tag{4}$$

where M_t and M_∞ represent the amount of solute released at time t and in equilibrium, respectively, the ratio M_t/M_∞ is the fractional released solute at time t, k is a constant dependent on the solvent/polymer and external conditions (such as pH, temperature, ionic strength, *etc.*) and n is the diffusional exponent that can be related to the drug transport mechanism. The use of Eq. (4) allows the mechanism for the solvent transport into the interior of the gel and/or for the solute release out of the 3D matrix to be evaluated. When n is around 0.5, the transport mechanism is controlled by Fickian diffusion (Ritger and Peppas, 1987). For $n = 1$, the transport mechanism is described for zero-order kinetics. In this case, the amount of released solute increases straightforwardly with the time. This fact is associated with the macromolecular relaxation of polymeric chains of the hydrogel matrix. When the value of n falls between 0.5 and 1.0, anomalous transport is observed, which results in simultaneous contributions of diffusion and macromolecular relaxation. Table 5 presents the values of n and k obtained from the curves shown in Fig. 9, after applying Eq. (4). The value of n was 0.93 in both studied temperatures (36.5 and 60 °C). Therefore, the CS releasing from the PEC2 matrix at pH 8.0, in both temperatures, is a contribution of the diffusion and hydrogel matrix relaxation processes, which characterize an anomalous transport. Furthermore, PEC2 presents a thermal-sensitivity property, since the k value at 60 °C was 3.8 times higher than that obtained at 36.5 °C (see Table 5).

The fraction of CS released from PEC2 can also be treated as a diffusion-partition phenomenon. The parameter α determines the extension of occurrence of this phenomenon, being this parameter obtained through the equation. (Reis et al., 2007)

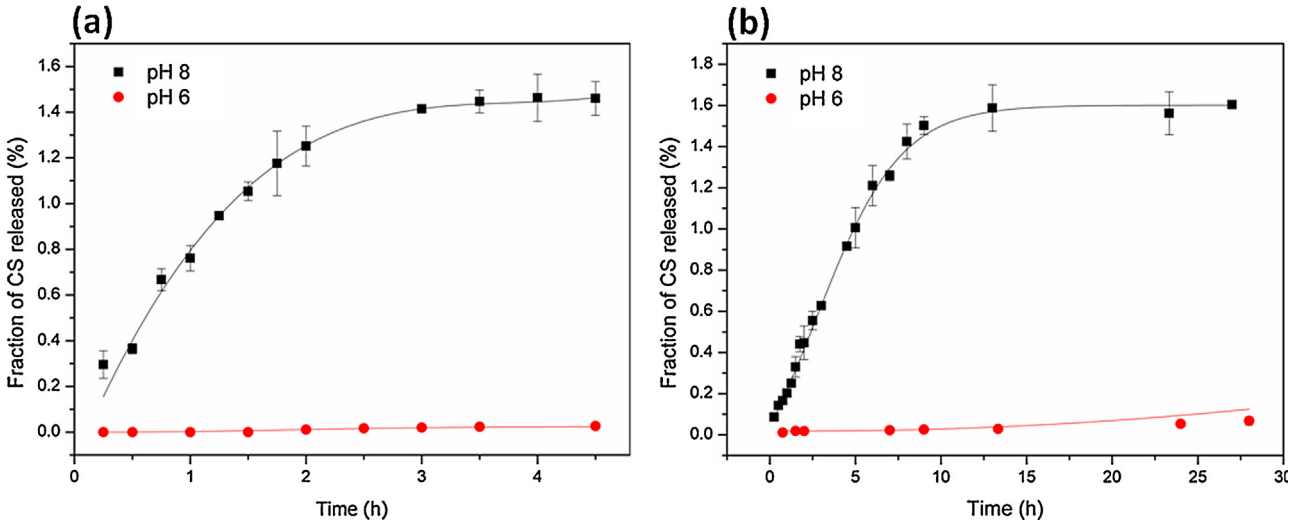

Fig. 9. Fraction (%) of CS released from PEC2 at 36.5 °C (a) and 60 °C (b).

Table 6
Value of α obtained from the Eq. (5) and k_r constant obtained by application of model described by the Eq. (6) on release curves of CS from PEC2 (Fig. 9) in mediums at pH 8.0 and different temperatures.

Temperature (°C)	A	$k_r(10^2)$	R^2
36.5	0.016	0.295	0.97
60	0.014	0.943	0.99

$$\alpha = \frac{F_{max}}{1 - F_{max}} \quad (5)$$

and the F_{max} is the maximum value reached for the fraction of released solute (Reis et al., 2007). The diffusion process depends on many variables, such as temperature, pressure, molecular size of the solute, concentration and swelling degree of the hydrogel. When the equilibrium is reached (as in Fig. 9, at pH 8.0), the rates of solute released and solute absorbed are equivalent and the fractional release (F_r) attains a maximum value (F_{max}) in that condition. In this way, the CS released from PEC2 could be treated as a diffusion phenomenon and/or partition phenomenon (Reis et al., 2007). After obtaining the values of F_{max} and F_r parameters from the release curves performed at pH 8.0 (Fig. 9), the respective kinetic constant of release (k_r) can be determined using the partition-diffusion mathematical model, Eq. (6), assuming that the CS release from PEC2 occurs according to second-order kinetics (Reis et al., 2007).

$$\frac{\alpha}{2}\ln\left(\frac{F_r - 2F_rF_{max} + F_{max}}{F_{max} - F_r}\right) = k_r t \quad (6)$$

The values of rate constant for the CS releasing (k_r) and α parameter are presented in Table 6. According to Ritger and Peppas, (1987), the semi-empirical relation (Eq. (4)) is valid only for a certain time interval, *i.e.*, the time in which up to 60% of the initial amount of solute is released (Ritger and Peppas, 1987). On the other hand, the diffusion-partition model based on Eq. (6) can predict the entire release of solute, *i.e.*, 100% (Reis et al., 2007).

From the analysis of Fig. S3 (Supplementary material) it can be seen that the models proposed by Eqs. (4) and (6) fit the experimental data presented in Fig. 9 in different way. For comparison purposes, the curves in Fig. S3 obtained by the application of Eqs. (4) and (6) were superimposed on experimental data obtained at pH 8.0 (Fig. 9). The prediction by Eq. (4) is not in accordance with the experimental CS release curves obtained at 36.5 and 60 °C (Fig. S3). On the other hand, the prediction by Eq. (6) is in good accordance with the experimental results, for 36.5 and for 60 °C as well (performed at pH 8.0). So, the release profile of CS from the PEC2 matrix at pH 8.0 can be better adjusted by the second-order kinetic model than by the empirical model given by Eq. (4).

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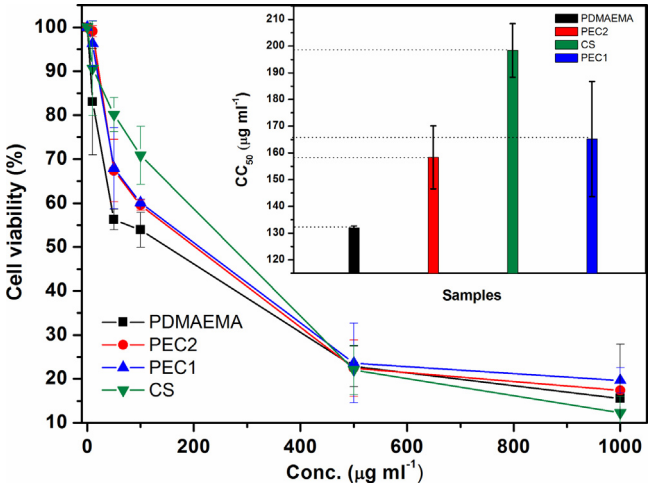

Fig. 10. Cytotoxicity effect of PDMAEMA, CS, PEC1 and PEC2 on the viability of VERO healthy cells at different concentrations. Error bars represent the standard deviation of three measurements.

3.10. Cytotoxicity assays

The cytotoxicity effects of CS, PDMAEMA, PEC1 and PEC2 systems were investigated through culturing healthy cells from the African green monkey (VERO cells). To assess the toxicity threshold for the four different materials, prepared as discussed in this work, viability cell assays were conducted through increasing concentrations of CS, PDMAEMA, PEC1 and PEC2 at 24 h incubation time, as shown in Fig. 10.

PDMAEMA acts more destructively against healthy VERO cells than CS, PEC1 and PEC2 samples (Fig. 10). The cytotoxicity effect of PDMAEMA on VERO cells was evidenced by the strong decrease in cell viability at concentration above 50 μg ml^{-1}. However, CS showed low (or almost no) cytotoxicity effect at concentration below 50 μg ml^{-1}. The half cytotoxicity concentration (CC_{50}) for PDMAEMA was 133 μg ml^{-1}, whereas the CC_{50} for CS was 198 μg ml^{-1}. So, the CS amount incorporated into the PEC matrix influences the cytotoxicity of the samples, since PEC1 presented CC_{50} = 166 μg ml^{-1} and the PEC2 sample showed CC_{50} = 157 μg ml^{-1}. Therefore, the higher the amount of CS in the PEC, the higher the CC_{50} against VERO cells. So, the presence of CS in PEC decreased the PEC cytotoxicity compared to PDMAEMA. This may potentiate the development of biomaterials based on this synthetic cationic polymer, which presents pH and thermo-sensitivity property. On the other hand, from the analysis of Fig. 10 one can verify the strong cytotoxicity effect of all samples at concentration above 400 μg ml^{-1}, since in all cases the cell viability decreased to c.a. 30% in these four studied samples.

4. Conclusions

Poly[(2-dimethylamino) ethyl methacrylate] (PDMAEMA) was prepared by radical polymerization of DMAEMA monomers, using AIBN as initiator. Polyelectrolyte complexes (PECs) based on PDMAEMA and chondroitin sulfate (CS) were obtained in different pH conditions, varying the PDMAEMA amount in each PEC formulation. The 2^2 factorial design with a central point indicates only the pH as a factor that significantly influences the amount of CS incorporated in PDMAEMA/CS matrices. The PEC1 and PEC2 samples presented the highest amount of CS incorporated. The properties of these formulations were further investigated using FTIR, TGA, SEM, WAXS and DLS. From DLS measurements it was verified that the PECs showed LCST in all studied pH conditions (6.0, 7.0 and 8.0) and this fact was attributed to the low mobility of PDMAEMA chains in the 3D matrix. On the other hand, PDMAEMA presented LCST at pH 8.0 but not at pHs 6.0 and 7.0. Moreover, at pH 8.0 the PECs showed LCST values below that of PDMAEMA. Release assays of CS from PEC2 showed that the release profiles depend on pH and temperature, with the CS release being more favoured at pH 8.0 (condition above the pK_a value of tertiary amine groups present in PDMAEMA chain segments) than at pH 6.0; and much more at 60 °C (condition above LCST) than at 36.5 °C. The diffusion-partition model (Eq. (6)) showed that the CS releasing from PEC2 proceeds via a second-order kinetic, being the releasing controlled by the partition-diffusion process. Cytotoxicity assays on VERO cells showed that CS increased the cell viability of PDMAEMA/CS PECs. These results open perspectives for preparing

and using new biomaterials based on PDMAEMA/CS indicating possible applications in the pharmaceutical and medical field.

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