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Article

Influence of the ionic strength on the physicochemical properties of methotrexate-loaded chitosan polyelectrolyte complexes

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Polyelectrolyte complexes (PECs) as drug delivery systems are widely explored since they are easily obtained by coacervation and biopolymers can be associated. However, particle distribution is a challenging critical parameter that has been infrequently focused. This work evaluated the effect of NaCl concentration on the physicochemical properties of PECs based on chitosan and hypromellose loaded with methotrexate. The particle size, zeta potential and polydispersity index (PdI) were determined by DLS, besides of drug entrapment efficiency (EE) and *in vitro* drug release profile determination. Particle size decreased while NaCl concentration rised, achieving a narrower size distribution of (345±79 nm) and PdI (0.285±0.067) with 200 mmol/L NaCl. The higher the NaCl concentration, the lower the zeta potential at acid pH. The EE was kept similar ((28.2±4.5) %) from 0 to 300 mmol/L NaCl, while 400 mmol/L NaCl impaired the drug entrapment. The addition of (200 and 300) mmol/L NaCl did not affect the drug release profile, but it was faster with (100 or 400) mmol/L. In conclusion, the addition of 200 mmol/L NaCl reduced particle size and PdI with no changes in the EE and drug release. Therefore, the ionic strength plays an important role on PECs development.

Keywords: Polyelectrolyte complex. Chitosan. Ionic strength. Polydispersity. Nanoparticles.

INTRODUCTION

The polyelectrolyte complexes (PECs) are particles composed by synthetic or natural polymers containing ionizable sites crosslinked by opposite charge ions or polymers. The polyelectrolyte complexation technique can offer interesting advantages such as ease and speed of execution, and harmful reaction conditions or sophisticated equipment are not required (Kulkarni *et al*., 2016). In this manner, PECs have been extensively used as micro and nanoparticles drug delivery systems. Nevertheless, the polydispersity and reproducibility of this method is a challenge to be overcome. These parameters are critical for stability control and scale up process, besides of biological implications such as direct influence on the absorption, distribution, and particle internalization by the target cells (Hoshyar *et al*., 2016).

Because polyelectrolyte complexation usually provides large size distribution particles, which is not desirable for drug delivery, methods to make less polydisperse PECs are necessary and must be developed according to each formulation. This work is focused on strategies for polydispersity improvement of chitosan PECs, which can be proposed as oral, mucosal, perioral, intralesional or intraarticular medicines. This polysaccharidic polymer is derived from chitin deacetylation, so it exhibits good biocompatibility and low cost. The chitosan structure is rich in amino groups, which can be protonated in acidic environment and become susceptible to interact with opposite charge groups

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(Shariatinia, 2019). There are many studies of chitosan associated with tripolyphosphate (TPP) PECs focusing on the narrow size distribution. The main reported variable parameters are: the chitosan molecular weight (Antoniou *et al.*, 2015) and deacetylation degree (Huang, Lapitsky, 2017), the concentration of chitosan and/or crosslinker parent solutions (Antoniou *et al.*, 2015; Huang, Lapitsky, 2017; Jonassen *et al.*, 2012), the chitosan/crosslinker ratio (Antoniou *et al.*, 2015; Barbi *et al.*, 2014; Jonassen *et al.*, 2012), the ionic strength (Antoniou *et al.*, 2015; Huang, Lapitsky, 2017; Jonassen *et al.*, 2012; Sawtarie *et al.*, 2017), and the pH (Antoniou *et al.*, 2015; Huang, Lapitsky, 2017).

Focusing on the ionic strength adjustment, the polydispersity control can be achieved by the electrostatic interactions slowdown between the cationic polymer and the polyanion, which allows the TPP solution disperses evenly when dropped into chitosan dispersion before the primary particles aggregate in larger particles (Huang, Lapitsky, 2017, 2011). It has been known that the ionic strength increment significantly reduced the link constant between chitosan and gum Arabic and k-carrageenan, which are anionic polymers (Rabelo *et al.*, 2019). In addition, it was demonstrated that the NaCl concentration increase from 0 to 300 mmol/L reduced the particle size and polydispersity index (PdI) of chitosan/casein complex (Ding *et al.*, 2019). Such findings encouraged us to test our hypothesis.

The commonest counter ion explored to obtain chitosan PECs is the TPP, an inorganic polyanion. Although TPP is usually used as crosslinker, PECs based on chitosan and other polymers, like hypromellose phthalate (HPMCP) (Boni *et al.*, 2018; Lacerda *et al.*, 2019; Pedreiro, Cury, Gremião, 2016), sodium carboxymethyl cellulose (Saha, Ray, 2013), sodium alginate (Saha, Ray, 2013; Taghe, Mirzaeei, 2019), and dextran sulfate (Gatti *et al.*, 2018), have also been developed as drug carriers. In contrast to the extensive reports on chitosan/TPP particle size control, approaches to decrease the chitosan/anionic polymer PEC polydispersity remain a field to be explored.

HPMCP is an anionic polymer commonly used as gastro resistant coating of oral dosage forms. HPMCP is an esterification product from hypromellose and phthalic anhydride, which attaches carboxyl groups in its structure (Shukla, Tiwari, 2012). Due to the electrostatic interaction possibility between carboxyl and amino groups, several kinds of drug delivery systems based on HPMCP and chitosan have been proposed: hydrogels (Akilo *et al.*, 2019; Mura *et al.*, 2018), micelles (Jaleh *et al.*, 2018), microspheres (Arthanari *et al.*, 2014), nanosuspension (Ambhore *et al.*, 2016), capsule (Verma *et al.*, 2017), oral tablet (Nunthanid *et al.*, 2008; Ruiz-Caro *et al.*, 2012), and vaginal tablet (Notario-Pérez *et al.*, 2018). This polymer was used in this work to obtain chitosan PECs.

In this work we have developed chitosan/HPMCP PECs as drug delivery system platform, and the methotrexate (MTX) was used as drug model. Exhibiting antitumor and anti-inflammatory properties, MTX is currently used as treatment for cancer, psoriasis and rheumatological diseases (Khan, Tripathi R, Mishra, 2012). Because of the ionizable amino and carboxyl groups presented in its molecular structure (Mioduszewska *et al.*, 2017), MTX is suitable to be easily incorporated to PECs.

The objective of this work was to explore the ionic strength influence on the polydispersity of MTX-chitosan/HPMCP PECs. The critical quality attributes measured were particle size, PdI, Zeta potential, encapsulation efficiency (EE%) and *in vitro* MTX release profile.

MATERIAL AND METHODS

Material

Low viscosity chitosan from shrimp shells (CAS number: 9012-76-4; molecular weight: 150 kDa; deacetylation degree>80%) was purchased from Sigma Aldrich (St. Louis, EUA). Methotrexate was from Fermion (OY, Finland). Dibasic sodium phosphate heptahydrate, and sodium hydroxide were from Vetec (Rio de Janeiro, Brazil). Potassium phosphate monobasic, and sodium chloride were from Dinâmica Química Contemporânea Ltda. (Indaiatuba, Brazil), and Labsynth (Diadema, Brazil), respectively. Acetic acid and hydrochloric acid were from Proquímios (Rio de Janeiro, Brazil). (HPMCP, type HP-55) was kindly donated from Shin-Etsu Chemical Co.® (Tokyo, Japan). The other reagents were of analytical grade. High-purity water was prepared with a Millipore® Milli-Q plus purification system.

PECs preparation

The chitosan particles were produced by polyelectrolyte complexation technique, adapted from the method reported by Pedreiro *et al.* (2016). A solution of 0.5 g/L methotrexate in 7.4 pH phosphate buffer solution was dropped on a 5.5 pH 4.0 g/L chitosan dispersion in 0.1 mol/L acetic acid solution, under stirring. Then, a 2.0 mg/mL HPMCP dispersion in 0.1 mol/L NaOH pH=5.5 solution was dropped on the first mixture. The system was kept under stirring for more 30 min. The ratio chitosan:HPMCP was 3:1 and the methotrexate concentration in relation to the polymers was 5%. The NaCl was included in the chitosan and HPMCP dispersion previously to the complexation step. The concentrations under evaluation were: 50 mmol/L (PEC50), 100 mmol/L (PEC100), 200 mmol/L (PEC200), 300 mmol/L (PEC300), and 400 mmol/L (PEC400), The PECs without NaCl were named PEC0. The fresh suspension of PECs was refrigerated for 24 h before the characterization procedures. Each sample was prepared at least four times and each time was considered a batch.

Particle size and PdI measurement

The size and PdI of the PECs were determined by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments, United Kingdom). All the samples were previously diluted (1:3.33) with purified water. The measurements were performed in triplicate, at the temperature of 25° C using the attenuation value of 8 and the detection angle of 173°.

Zeta potential titration

Zeta potential value according to the pH was achieved by using MPT-2 autotitrator attached to Zetasizer Nano ZS. The pH ranged from 3 to 10 (each pH point was read in triplicate), and 0.5 mol/L HCl, 0.5 mol/L NaOH, and 0.25 mol/L NaOH aqueous solutions were used as titrants. The samples were previously diluted to 1.0 mg/mL with purified water. Each titration was performed using a pool of 4 batches for sample.

Drug entrapment efficiency (EE)

The EE was indirectly determined from free drug quantified in the PECs suspension (Pedreiro, Cury, Gremião, 2016)**.** In order to quantify the free MTX in all the samples, 1 mL of PECs suspension was centrifuged at 12,000 x *g* for 1.5 h. Two hundred microliters of the supernatant were diluted to 1 mL with 0.02 M acetate buffer solution pH 4.3. After filtering through 0.45 µm pore membrane, the samples were analysed by HPLC with a Prominence apparatus (Shimadzu®, Kyoto, Japan). A C18 end-capping column 150.0 x 4.6 (i.d.) with 5 µm particle size (LiChrospher® 100 RP-18 end capped Merck) was employed. A mixture of 4.3 pH 0.02 mol/L acetate buffer solution:methanol:acetonitrile 77:16:7 (volume by volume) was used as mobile phase and flowed at 1.0 mL/min. The UV detection was carried out at 306 nm. Data from the analytical method validation are shown as supplementary material.

The EE was calculated as:

$$EE\ (\%) = (total_{MTX} - free_{MTX}) / (total_{MTX}) \times 100$$

In vitro drug release

The MTX release from the PECs was investigated using Franz-type diffusion cells (Hanson Research Corporation, USA) covered with a regenerated cellulose acetate dialysis membrane (Spectra/Por, 12-14 KDa cut-off) (Mendes *et al.*, 2019). A 6.8 pH solution containing 0.80% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, and 0.02% KH_2PO_4 w/v (Marques, Loebenger, Almukainzi, 2011) was used as receptor medium. The assay was performed in triplicate at temperature of 37° C, 300 rpm of stirring, under assured sink conditions. Aliquots were withdrawn after 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h, and they were analysed by HPLC according to the conditions described in the previous section. The results were fitted to First Order, Higuchi, Korsmeyer-Peppas and Weibull models by no linear regression using Sigma Plot software (SYSTAT SOFTWARE Inc, 2006). The best kinetic model was chosen according to the highest adjusted coefficient of determination (($R^2_{adjusted}$)) and the

lowest Akaike Information Criterion (AIC) values, which was calculated by the following equation (Costa, Lobo, 2001): $AIC = n \times ln(WSSR) + 2 \times p$, where n is the number of release data points, p is the number of the parameters of the model, and $WSSR$ is the weighed sum of square of residues. From the best fitted mathematical model, the times enough to release 30%, 50%, and 80% of MTX were achieved ($t30\%$, $t50\%$, and $t80\%$).The PEC50, PEC100, PEC200, PEC300, and PEC400 release profiles were compared to PEC0 one by the difference factor ($f1$) and the similarity factor ($f2$), which were calculated by the following equations (Costa, Lobo, 2001):

$$f1 = \frac{\sum_{j=1}^{n}|R_j - T_j|}{\sum_{j=1}^{n}\frac{(R_j+T_j)}{2}}, f2 = 50 \times log\left\{\left[1 + \frac{1}{n}.\sum_{j=1}^{n}|R_j - T_j|^2\right]^{-0,5} \times 100\right\},$$

where n is the number of release data points, R_j and T_j are the percent of released drug of PEC0 and test PEC at each time point j. If $f1>15$ and $f2<50$ the drug release profiles were regarded as different.

Statistical analysis

Descriptive measures were used in order to express the results of size, PdI, and EE measurements. Kruskal Wallis analysis was applied in order to compare the groups, using Agricolae package (De Mendiburu, 2017) from R software (R CORE TEAM, 2018).

RESULTS AND DISCUSSION

Particle size, PdI, Zeta potential and EE

The results of particle size are exhibited in Figure 1A.

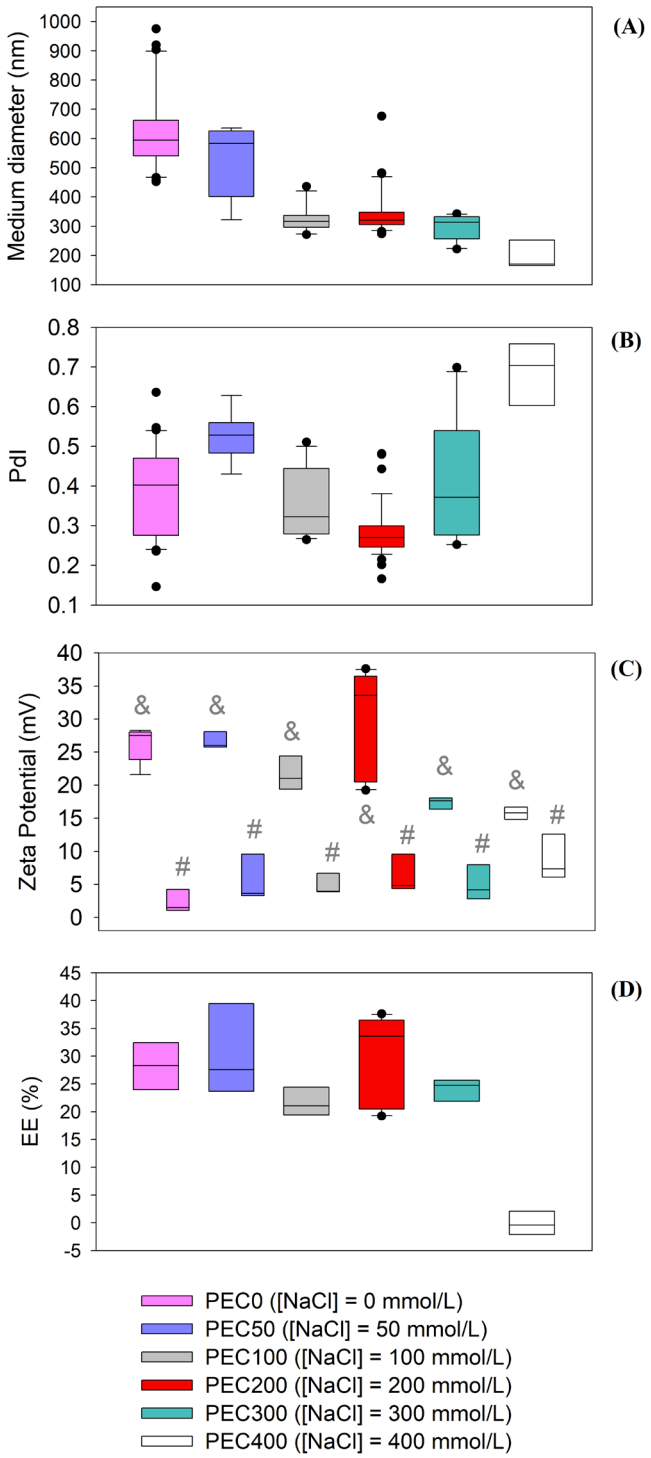

FIGURE 1 - Effect of NaCl concentration on particle size (A), polydispersity index (PdI) (B), Zeta potential (C) and drug entrapment efficiency (EE) (D). The data are presented as median, interquartile range, minimum and maximum values. Dots indicate outlier values. & indicates Zeta potential measure at pH 5.6-5.8; # indicates Zeta potential measure at pH 7.2-7.4.

It was noted that the addition of NaCl decreased the size particle from 625 nm ± 137 nm (PEC 0) to 197 nm ± 56 nm (PEC 400). This behaviour corroborates to those from other authors (Antoniou *et al.*, 2015; Jonassen *et al.*, 2012; Yang *et al.*, 2018). In contrast, larger sizes were also observed on account of raising the ionic strength (Antoniou *et al.*, 2015; Huang, Lapitsky, 2011; Sawtarie *et al.*, 2017). Besides ionic strength, other parameters like the deacetylation degree of chitosan (Ding *et al.*, 2019; Sawtarie *et al.*, 2017), the concentration of the starting material solutions (Antoniou *et al.*, 2015; Yang *et al.*, 2018), the chitosan/crosslinker rate (Antoniou *et al.*, 2015; Huang, Lapitsky, 2011), and pH (Antoniou *et al.*, 2015; Ding *et al.*, 2019) can impact on particle size, polydispersity and/or zeta potential. Therefore, changing such conditions can produce different results.

One of the reasons appointed for explaining smaller sizes in saline conditions refers to the chitosan chain conformation. In aqueous medium, in the absence of salt, the protonated amino groups repel each other and lead to a more extended and stiff polymeric chain. When a monovalent salt is dissolved in the solvent, the saline ions shield the chitosan cationic sites reducing the electrostatic repulsion among them. For this reason, the polymeric chains become more flexible, assume a coil shape and provide obtention of smaller particles (Jonassen *et al.*, 2012).

Another fact that could be related to the particle size is the difference of osmotic pressure between the PEC's outside and inside (Ding *et al.*, 2019; Yang *et al.*, 2018). When the PECs are obtained in saline conditions, the NaCl concentration inside the PEC is almost the same of that in the dispersion medium (Yang *et al.*, 2019). Since the osmotic pressure is balanced, the propensity of particle swelling is lower.

The addition of NaCl also modified the PdI (Figure 1B), nevertheless an exact correlation was not found. PEC200 exhibited the lowest PdI. The decrease on PdI with determined electrolyte concentrations has been mainly attributed to a lower aggregation induced by bridging (Huang, Lapitsky, 2017, 2011). When the ionic strength is increased, the enthalpy of PEC formation becomes less exothermic because the electrostatic attraction is reduced since the polyelectrolytes are shield by counter ions (Fu, Schlenoff, 2016). The presence of the saline ions weakens the interaction between chitosan and TPP because Cl^- competes with TPP ions for the cationic sites (Sawtarie *et al.*, 2017), therefore the polyelectrolyte complexation occurs more slowly in a such way that the aggregation between the primary particles is prevented. It has been already noted that when the ionic strength increased from (0 to 100) nmol/L NaCl, the coupling constant between chitosan and anionic polymers (gum Arabic and k-carrageenan) reduced (Rabelo *et al.*, 2019). Thus, it would be reasonable to assume that NaCl could decrease the interaction between chitosan and HPMCP, which is a polysaccharide rich in carboxyl groups like gum Arabic. This hypothesis could probably explain the reduced PdI exhibited by PEC 200 in this work. The decreased particle formation rate could allow the dropped HPMCP dispersion mix more homogeneously into the chitosan dispersion before significant aggregation can occur (Huang,Lapitsky, 2011; Lapitsky, 2014). Concentrations bellow 200 mmol/L NaCl were not enough to achieve such effect. On the other hand, above 200 mmol/L NaCl the PdI raised significantly. In the cases of PEC300 and mainly PEC400 the interaction between the polymers was so undermined by the high NaCl content that the particle formation was presumably impaired. The damage to chitosan nanoparticles formation due to high electrolytes concentrations has been also reported by other authors (Huang, Lapitsky, 2017, 2011; Sawtarie *et al.*, 2017).

The effect of NaCl addition on Zeta potential depends on the pH range analysed (Figure 2). At the pH in which the PECs are obtained (Figure 1C, indicated by &), when the NaCl concentration was increased the Zeta potential was reduced, and this parameter ranged between +31 mV and +15 mV, that means for all the samples the original Zeta potential is enough to give the PECs medium colloidal stability (Salopek *et al.*, 1992). The Zeta potential decrease caused by NaCl increase was also noted by Jonassen *et al.* (2012) for PECs based on chitosan:TPP (4:1). Such effect became more pronounced at lower pH. However, under physiologic conditions, around pH 7.4, the opposite trend was observed (Figure 1C, indicated by #), the addition of NaCl increased the Zeta potential, whose maximum value was +7.9 mV. The Zeta potential value for PEC0 at pH 5.5 was near to that

from previously reported MTX-loaded PECs based on chitosan and hyaluronic acid, +29.6 mV, which decreased to +20.9 mV when HPMCP was incorporated to the particles (Boni *et al.*, 2018).

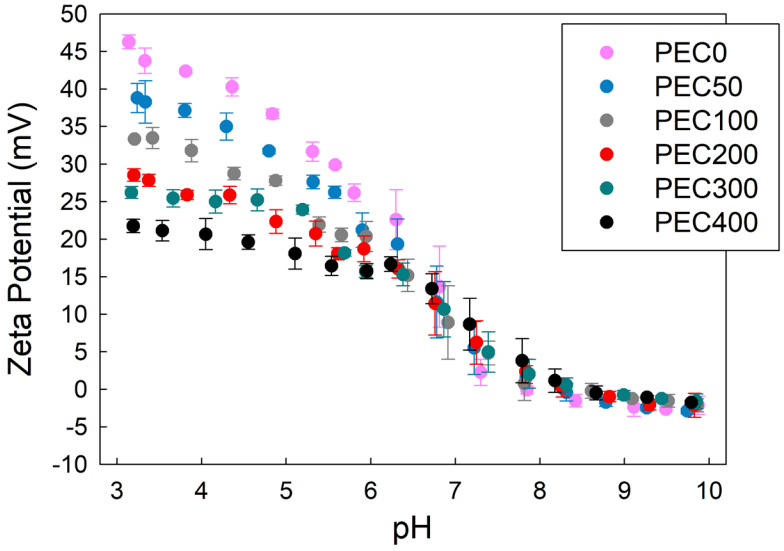

FIGURE 2 – The effect of NaCl concentration on the Zeta potential according to the pH.

The NaCl addition kept the EE around 25-30% (Figure 1D), without changing such parameter in relation to PEC0, except for 400 mmol/L, when a sharp decrease was noted. Presumably, in PEC400 the ionic strength was so high that it prevented suitable particle formation and the MTX was not encapsulated. Thus, by using until 300 mmol/L NaCl, it was possible to keep the EE. The found EE was coherent with other MTX-loaded chitosan PECs previously reported, 30.0-37.4% (Boni *et al.*, 2018), although the particle composition was not the same.

In vitro drug release

The MTX release profile from all the PECs (Figure 3A) best fitted to Weibull's mathematical model according to the highest and lowest AIC (Table I).

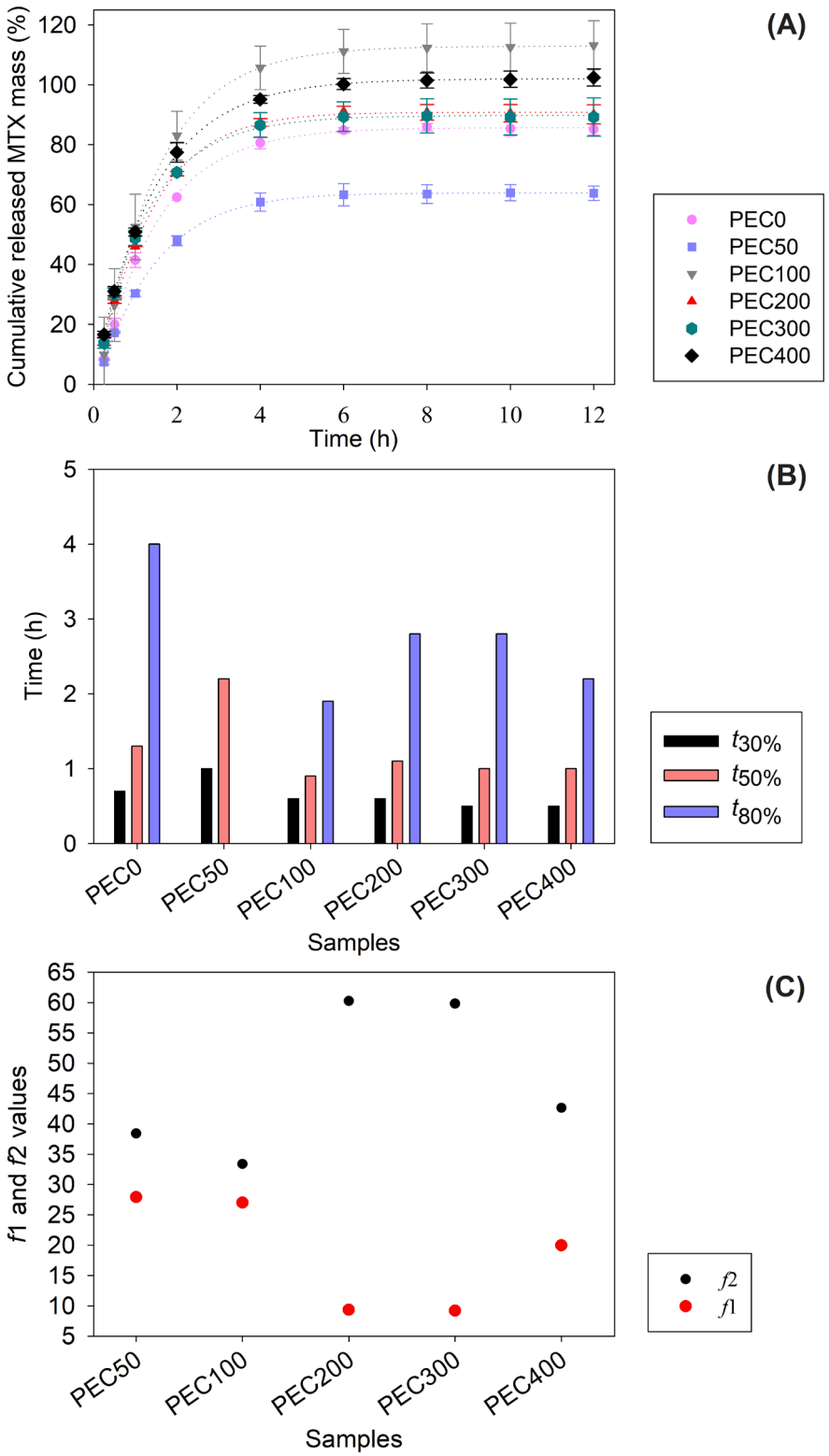

FIGURE 3 – (A) Drug release profiles from PECs containing different NaCl concentrations; (B) Enough time to release 30%, 50%, and 80% of methotrexate; (C) Difference factor (f1) and similarity factor (f2) of the PECs containing NaCl in relation to PEC0.
Legend: In (A), the dotted lines indicate the fitting to Weibull's mathematical model

TABLE I - Adjusted coefficient of determination ($R^2_{adjusted}$) and Akaike Information Criterion (AIC) values provided by the in vitro drug release data fitted to different mathematical models

Sample	First Order		Higuchi		K.-Peepas		Weibull	
	$R^2_{adjusted}$	AIC	$R^2_{adjusted}$	AIC	$R^2_{adjusted}$	AIC	$R^2_{adjusted}$	AIC
PEC0	0.9172	59.98	0.9329	54.36	0.9164	56.34	0.9991	20.83
PEC50	0.5682	69.16	0.9352	48.45	0.9194	50.40	0.9996	7.84
PEC100	0.9361	62.76	0.9295	59.87	0.9124	61.81	1.0000	-6.22
PEC200	0.9585	53.54	0.9342	54.24	0.9263	55.26	0.9993	18.28
PEC300	0.9378	56.59	0.9137	56.19	0.9120	56.36	0.9991	20.71
PEC400	0.9982	27.17	0.9358	55.49	0.9306	56.20	0.9997	13.16

Foot note: K.= Korsmeyer

The initial burst effect of releasing from PEC50 was lower than from the other PECs, as showed by the highest $t_{30\%}$ and $t_{50\%}$ and the MTX release from PEC50 did not achieve 60% up to the end of the assay (Figure 3B). The MTX release from PEC100 and PEC400 was faster than the other samples. Whereas PEC50 exhibited $t_{50\%}$ of 2.2 h, PEC100 and PEC400 demonstrated $t_{80\%}$ of 1.9 h and 2.2 h, respectively. The highest $t_{80\%}$ (4 h) was found for PEC0. Even though PEC200 and PEC300 exhibited t80% of 2.8 h, the MTX release profile from these samples were not significantly different in relation to that showed by PEC0, as can be observed by $f1<15$ and $f2>50$ (Figure 3C). In contrast, the values of f1 and f2 showed that the use of (50, 100, or 400) mmol/L NaCl really modify the MTX release profile in relation to PEC0. The modification observed in PEC50 was not desirable because the MTX release was impaired. The MTX release from PEC100 and PEC400 occurred in a shorter time period, which could be desirable in drug delivery systems for immediate release or unsuitable when a sustained release is worthwhile.

The drug release from PECs can be performed if the polymeric matrix is eroded by chemical or enzymatic action and/or by decoupling of the polyelectrolyte interaction (Lapitsky, 2014). The second reason mentioned above was the mechanism involved in the *in vitro* drug release assay carried out here, since there was not any erosive condition to the particles because the temperature and receptor medium used in the assay were not deleterious to the PECs.Our results indicated that, although the addition of NaCl (up to 300 mmol/L) did not change the amount of drug loaded into the PECs, the monovalent salt could modify the rate of MTX release depending on NaCl concentration, such as observed for PEC50 and PEC100 compared to PEC0. PEC0 and PEC50 exhibited higher particle size, that means lower interfacial area, and the drug release from these PECs were slower, resulting in higher $t_{50\%}$ values. Nevertheless, by analyzing f1 and f2, it can be noted that the MTX release profile from PEC0 is equivalent to that one exhibited by PEC200 and PEC300, which are smaller particles than PEC0. No obvious correlation between size particle, Zeta potential and PdI with the drug release profile was found.

CONCLUSION

The addition of electrolytes enabled to improve features such as size, PdI, maintaining the EE%, and it can modify the drug release profile. Therefore, it is an input parameter that must be considered when developing PECs in order to adjust critical quality attributes (CQAs) such as size, polydispersity and drug release profile.

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