Preparation of alginate-chitosan polyelectrolyte complexes for encapsulation of natural polyphenols

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Chitosan-alginate polyelectrolyte complexes (PECs) for polyphenols encapsulation using chitosan polymer and pre-gelated sodium alginate were prepared. Natural polyphenols entrapped in PECs were extracted from rose hips (*Rosa canina*) with a total phenolic content of 15.8 mg/mL Galic acid equivalents (GAE). The effects of polyphenols concentration and pH on the particle size, zeta potential and release rate were studied. Polyphenol-polyelectrolyte interactions were investigated by Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC). Dynamic light scattering (DLS) analysis showed an average particle size ranged between 962 nm – 2384 nm. Furthermore, the delivery profiles of polyphenols from PECs were assessed.

(Received July 19, 2015; accepted February 10, 2017)

Keywords: Alginate, Chitosan, Polyelectrolyte complexes, Polyphenols

1. Introduction

Nanoscale/Microscale drug delivery systems obtained from biocompatible and biodegradable polymers constitute one of the most attractive areas of research. The uses of biodegradable polymers as drug carriers have been of interest in controlled-release technology since these polymers are easily metabolized in the body. As natural biomaterials, alginate (ALG) and chitosan (CS) are polysaccharides that are highly stable, safe, non-toxic, hydrophilic and biodegradable [1].

Oppositely charged polysaccharides in aqueous solutions interact spontaneously to form polyelectrolyte complexes (PECs) when they are mixed [2]. Interactions between cationic chitosan and anionic alginate lead to PEC formation, and these PECs have potential applications such as drug [3] or gene delivery systems in biomedicine [4].

Chitosan is a linear binary copolymer that consists of β (1→4)-linked 2-acetoamido-2-deoxy-β-D-glucopyranose (Glc-NAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit). The A- and D-type residues are randomly distributed along the chitosan chain [5]. The relative amounts of acetylated groups can vary depending on the source and methods of processing. The primary amine groups render special properties that make chitosan very useful in pharmaceutical applications [6].

Alginate is a linear copolymer composed of β-D-mannuronic acid and α-L-guluronic acid joined by a 1-4 glycosidic bond. The composition and patterning of the monomers are dependent on the source of the polysaccharide. The most common source of alginate is the cell wall of brown algae. Alginate is known as biocompatible, biodegradable and non-toxic polymer and has shown great promise in biomedical applications due to the reactivity of its carboxylate side groups and its capacity to form spontaneous gelation when exposed to divalent cations such as calcium [7]. Recently, the PECs between ALG and CS has been used for tissue engineering [8] and specific drug delivery [9].

Phenolic compounds are considered as secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation, among others [10]. Plant phenolics include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins [11]. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer [12]. Unfortunately, these properties are also responsible for a lack in long-term stability, making these natural compounds very sensitive to light and heat. Moreover, polyphenols often present a poor biodisponibility mainly due to low water solubility. Lastly, many of these molecules possess a very astringent and bitter taste, which limits their use in food or in oral medications [13].

The objective of this study was to evaluate the possibility of obtaining CS-ALG PECs as carriers for natural polyphenols. A two-stage procedure was used to form PECs. The pH of the polyelectrolytes was chosen considering that the interaction between alginate and chitosan was reported to be pH dependent and stronger complexes were obtained at pH around 4.5-5 [14]. Chitosan has the limited solubility in the higher pH region, gelation or precipitation occurs where the pH exceeds 6 and alginate precipitation was observed below ~ pH 3.6.

The observed pH values causing precipitation correlate well with the published pK values of these polyions. The alginate chains are composed of mannuronic and guluronic acid units whose pK values are published as 3.38 and 3.65, respectively. The chitosan chain pK is known to be around 6.3 [15]. The pH has a strong influence on polyelectrolyte functional groups. As the pH increased the number of dissociated carboxylic groups in alginate increased and reversely the number of protonated amine groups in chitosan decreased. At the extremely lower pH region, most carboxylic groups of alginate are in the form of COOH. In contrast, at the extremely higher pH region, most amine groups of chitosan are in the form of NH_2. However, PECs can be obtained by the induced dissociation of carboxylic or amine groups with coexisting polyions of opposite charge [16].

Polyelectrolyte complexes of sodium alginate with chitosan were developed for the encapsulation of high-value bioactive molecules as phenolic compounds. Thus, a yerba mate (*Ilex paraguariensis*) freeze-dried extract was encapsulated [13]. In this research, the release of gallic acid shows the importance of the nature of the wall material on the release of natural polyphenols present in yerba mate. Another study highlights the formation, characterization and application of PECs as a potential carrier to improve both the solubility and the biological activity of curcumin [18].

Numerous articles have been published regarding chitosan–alginate systems [17, 18] but few studies have been done to compare the properties of complexes formed between chitosan and alginate with and without natural polyphenols.

The interactions between components of encapsulated rose hips extract were studied through FTIR and DSC. The effects of polyphenols concentration, alginate-chitosan mass ratio and pH on the particle size, zeta potential, and the release properties of PECs in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 7.4) were studied.

2. Experimental

2.1. Materials

Alginic acid sodium salt from brown algae bio-reagent and Chitosan of medium molecular weight, derived from crab shell were purchased from Sigma-Aldrich Chemie UK. Rose hips *(Rosa canina)* were acquired from the local pharmacy and then pulverized and stored in a desiccator before extraction and analysis. Ethanol, Folin-Ciocalteau phenol reagents were provided by Merck. Gallic acid was also purchased from Sigma-Aldrich. All the other reagents used in the experiments were of analytical grade.

2.2. Extraction of polyphenols from rose hips

Dried rose hips (10 g) were sonicated for 30 minutes at room temperature in ethanol: water (50:50 v/v). The extract was centrifuged at 4000 RPM for 20 minutes. The residue was re-extracted by repeating the procedure mentioned above. The 50 % aqueous ethanol combined supernatants were evaporated at 35 ^{0}C under vacuum to remove solvents. The polyphenols extract was concentrated up to 20 % of solid content was achieved.

2.3. Preparation of chitosan alginate PECs

Both sodium alginate and calcium chloride solutions were prepared by dissolving the required amount of alginate/ calcium chloride in distilled water. The pH of sodium alginate was adjusted to 5.1 using hydrochloric acid. A known amount of chitosan was dissolved in 1% acetic acid solution and the pH was adjusted to 5.4 using NaOH 1M. The method used to prepare the chitosan-alginate PEC is a two-step method adapted by P.Li [19] from M. Rajaonarivony [20]. The pH of alginate and chitosan solutions was set to 4.9 and 4.6 to obtain the PEC with pH of 4.7.

Preparation of blank chitosan-alginate PECs

Aqueous calcium chloride (2 ml of 3.35 mg/mL) was added dropwise to 10 mL aqueous sodium alginate (3.0mg/ml) while stirring for 30 min (1200 RPM), and then 4 mL chitosan solution (0.8 mg/mL) was added into the resultant calcium alginate pre-gel and stirred for an additional 1 hour. The resulting opalescent suspension was equilibrated overnight to allow PECs to form uniform particle size.

Preparation of polyphenols loaded chitosan-alginate PECs

Different CS-ALG PECs formulations were prepared by varying the polyphenols concentration (0.25 mL – PECTP0.25; 0.5 mL - PECTP0.5 and 1 mL - PECTP1). The total phenolic content (TPC) of rose hips extract was of 15.8 mg/mL. Polyphenols were loaded by incorporating the specified volume of polyphenols extract into calcium chloride solution. Then, the procedure was the same as for the preparation of blank CS-ALG PECs.

2.4. Characterization of PECs

Particle size and zeta potential analysis

The measurements of particle size and zeta potential of PECs were performed on a Zetasizer Nano-ZS on the basis of Dynamic light scattering (DLS) techniques. Based on measurements of the Brownian motion of the particles in the solution, the average diameters of the particles were determined using Stokes-Einstein equation.

Fourier Transform Infrared spectroscopy (FTIR) analysis

The PECs were freeze-drying (Martin Christ - Alpha 1-2 Plus) and then analyzed as specified below.

Both biopolymers standards and lyophilized CS-ALG PECs with polyphenols were investigated with FTIR Spectrum GX Perkin Elmer equipment. The samples were

analyzed in KBr by transmission with 64 scans per experiment.

DSC analysis

Termograms were obtained using DSC 823 system (Mettler Toledo). The lyophilized samples, ~5 mg (polymer standards/ prepared PECs) were heated from 40-400 ^{0}C with a heating constant rate of 10 ^{0}C/min under constant purging of nitrogen.

2.5. In vitro release studies

The polyphenols release from PECs was studied by incubating 5 mg of PECs in 5 mL simulated gastric fluid, SGF (pH=1.2), and 5 mL simulated intestinal fluid, SIF (pH = 7.4) at 37 ^{0}C and 100 RPM/min. At fixed intervals of time 1 mL of sample was withdrawn and replaced with the same amount of fresh medium. The total phenolic content in the release medium was measured using the method applied by [21], after centrifugation at 4000 RPM and 4 ^{0}C for 30 min. Supernatant of empty PECs was taken as blank.

$$Released\,rate,\% = \frac{Released\ polyphenols}{Total\ polyphenols} \cdot 100 \quad (1)$$

Release kinetics

The drug release data PECs was fitted to the Korsmeyer-Peppas model [22] only up to ~ 60% cumulative drug release. Mathematical model used to ascertain polyphenols release:

$$\frac{Q_t}{Q_\infty} = K_k t^n \quad (2)$$

$\frac{Q_t}{Q_\infty}$ is the fraction of polyphenols released in time *t; n* is the release exponent, indicative of the mechanism of polyphenols release and K_k is Korsmeyer-Peppas constant, respectively, determined by fitting the release data in equation (2).

3. Results and discussion

3.1. Particle size and zeta potential

The effect of polyphenols concentration on the particle size shows (Fig. 1) an increase of size from 1261 to 2384 nm with decreasing polyphenol concentration from 15.8 mg/mL to 3.9 mg/mL. The zeta potential values decreased from -29.1 mV (PECTP1) to -49.2 mV (PECTP0.25). These observations indicated that the polyphenols concentration affects the size and zeta potential of PEC particles produced.

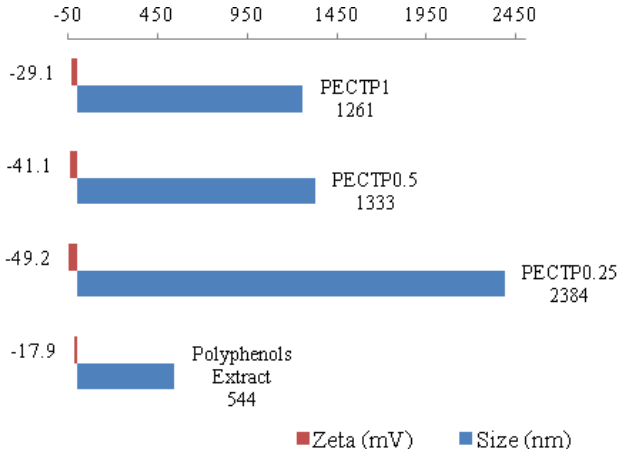

Fig. 1. Particle size and zeta potential analysis by polyphenols concentration

The formation of the alginate-chitosan particles was influenced by alginate: chitosan mass ratio and pH. Alginate-chitosan polyelectrolyte complexes at two pH values were analyzed by DLS. The results obtained, show that pH 4.7 was the most appropriate for the PEC formation since the smallest size was observed for this pH. At pH 4.7 the carboxyl groups of the ALG are ionized and the amine groups of CS are protonated which is the most important for optimum interaction and the PEC formation [14].

The decrease of pH from 5.2 to 4.7 slightly decreased the mean particle size of obtained PEC from 1261 nm to 962 nm. Also, the zeta potential values decreased from -29.1 to -36.2 mV. At a pH 5.2, CS approaches its pKa value (~ 6) and precipitation may occur, resulting less CS available for PEC formation [23].

The effect of alginate to chitosan mass ratio on particle size showed an increase of size from 962 nm to 2207nm with decreasing alginate to chitosan mass ratio from 9.3:1 to 6:1. These results correspond with earlier reported by B. Sarmento [24].

3.2. Fourier transform infrared spectroscopy evaluation

FTIR spectra of NaALG, CS, blank ALG/CS PEC and polyphenols-loaded PECs with the specific absorption bands are presented in Fig. 2. Because is known that the carboxyl group (COO^-) of the anionic polymer may interact with the amino group ($-NH_3^+$) of chitosan to form an ionic complex, changes in the absorption bands of the amino group, carboxyl group, and amide bonds were evaluated.

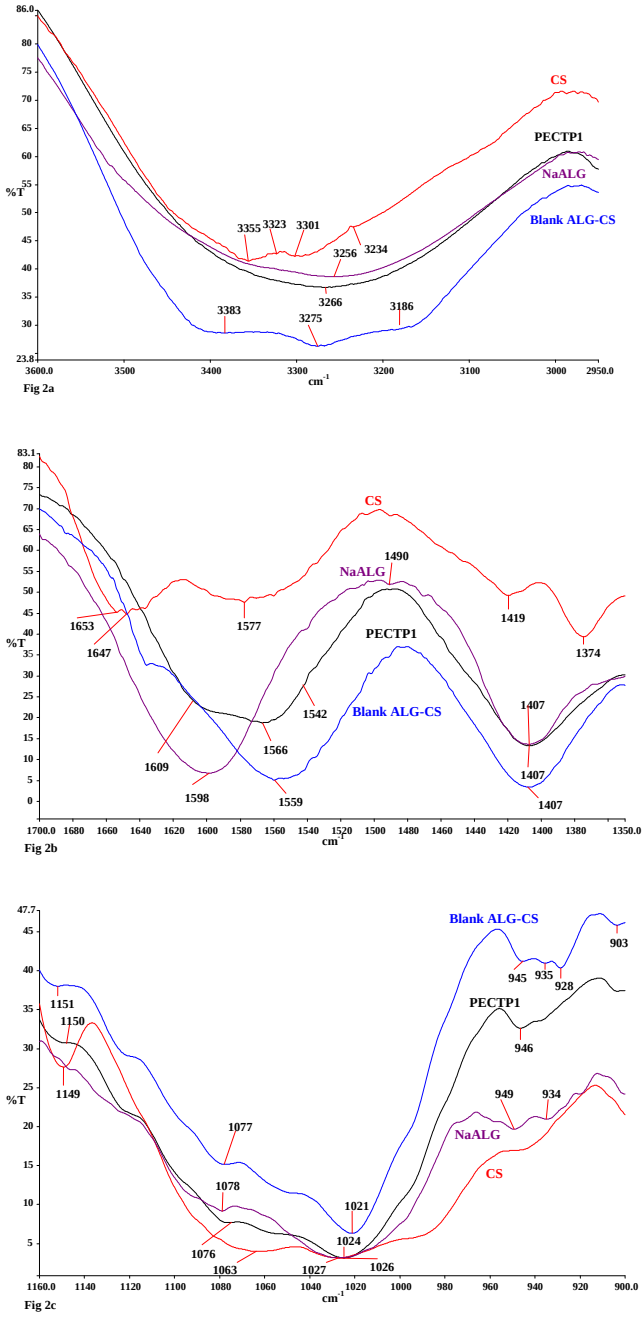

Fig. 2a, 2b, 2c, of FTIR spectra, on different spectral range: CS, NaALG, Blank ALG-CS, ALG-CS PECs loaded with polyphenolic natural extract

In the CS spectrum, the broad band at 3355 cm^{-1} corresponds to the amine N-H and hydroxyl (OH) groups in Fig. 2a. The bending vibration of the N-H (amide II band) was observed at 1577 cm^{-1}. The bands at 1592cm^{-1} and 1412 cm^{-1} in the NaALG spectrum were assigned as asymmetric and symmetric stretching peaks of carboxylate salt (COO^-) groups in Fig. 2b.

After loading of polyphenols was observed that the asymmetric stretching of the (COO^-) groups and the amide band of CS shifts to 1566 cm^{-1} after the reaction with alginate in Fig. 2b.

For PECTP1 sample, the 3266 cm^{-1} O-H stretching band became slightly narrower, probably due to the breaking of some hydrogen intermolecular interactions, in Fig. 2a.

The most important change on the spectra of the polyelectrolyte complexes containing polyphenols was the shoulder observed between 1566 cm^{-1} and 1542 cm^{-1}, assigned to the interaction of different polar groups of the active compound with the hydroxyl or carboxylate groups of the alginate, in Fig. 2b, or to interactions between hydroxyl/carbonyl/aldehyde groups of the extract to the amine groups of the chitosan.The structure of phenolic acids by FTIR, indicating that the peak at 1607 cm^{-1}, corresponded to the -CO-C=C- group, the emerging peak could be explained by the interaction between this group and chitosan.

Other hydroxyl-related bands were observed between 1150 cm^{-1} and 1067 cm^{-1} and 946 cm^{-1} in Fig. 2c.

3.3. Thermal analysis

The differential scanning calorimetry (DSC) method was applied to study the thermal behavior of biopolymers and to explain the interaction between chitosan, alginate, and polyphenols. The results obtained by DSC samples are presented in Table 1.

Table 1. DSC analysis of CS, NaALG, Blank and PECs

Compound	Attribution	DSC	
		peak/^{0}C	
NaALG	DEH	95.2	(endo)
	DEC	252.0	(exo)
CS	DEH	88.3	(endo)
	DEC	223.8	(endo)
PECTP0.25	DEH	51.7	(endo)
		132.9	(endo)
	DEC	153.6	(endo)
		314.7	(endo)
PECTP 0.5	DEH	131.5	(endo)
	DEC	183.6	(endo)
	DEC	297.4	(exo)
PECTP 1	DEH	131.6	(endo)
	DEC	187.1	(endo)
	DEC	294.0	(exo)
DEH- dehydration; DEC - decomposition			

The endothermic peak for chitosan at 88.3 ^{0}C can be ascribed to the loss of water and the second thermal event may be related to the decomposition of amine units [25] with a correspondent exothermic peak at 223.8 ^{0}C.

Thermogram of sodium alginate showed an endothermic peak 95.2 ^{0}C due to removal of absorbed

moisture and an exothermic peak at 252.0 ^{0}C obtained as a result of decomposition.

The polyphenols extract showed an endothermic peak at 53.2 ^{0}C and multiple endothermic peaks in the temperature range between 120.0-180.1 ^{0}C which can be attributed to the numerous compounds present in the extract.

As can be seen in Table 1 the endothermic peaks are correlated with loss of water associated to hydrophilic groups of polymers while exothermic peaks resulted from degradation of polyelectrolytes due to dehydration and depolymerization reactions most probably to the partial decarboxilation of the protonated carboxylic groups and oxidation reactions of the polyelectrolytes [14].

It was observed that the endothermic and exothermic peaks of the complexes (PECTP0.25, PECTP0.5, PECTP1) shifted from those of blank or biopolymers which were interpreted as an interaction between components.

The exothermic peaks of PECs registered at 297.4 ^{0}C and 297.0 ^{0}C which are higher than the peak value of alginate and chitosan, indicating the stronger interaction between biopolymers and polyphenols.

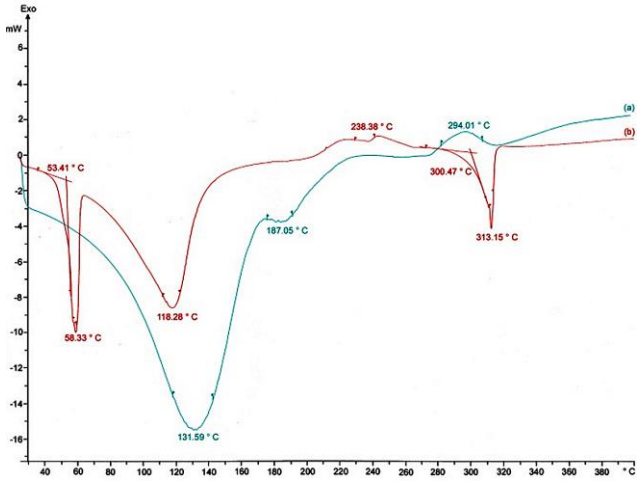

Fig. 3. DSC analysis of blank chitosan-alginate PECs (b) and ALG-CS PECs loaded with polyphenolic natural extract, PECTP1(a)

The endothermic peak around 131.6 ºC shifted from those of blank which became very broad (Fig. 3), may be assigned to the encapsulated polyphenol extract. The endothermic peak registered at 294 ºC which was absent from either the chitosan or alginate thermogram, could be ascribed to the formation of an ionic pair between the carboxylate group (-COO^-) of alginate and the ammonium group (-NH_3^+) of chitosan [26].

3.4. In vitro release studies

The content of total phenols was determined, from calibration curve (Y = 0.004X +0.0018) and r^2 = 0.9994, as gallic acid equivalents (GAE).

In vitro polyphenols released of prepared PECs was carried out both in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

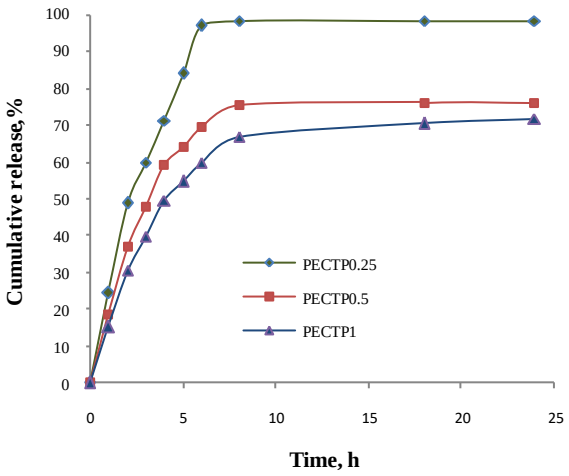

Fig. 4. The cumulative release curves of polyphenols from CS-ALG PECs at pH 7.4 and 37^{0}C

The cumulative curves of CS-ALG PECs at pH 7.4 and 37 ^{0}C as a function of time are presented in Fig. 4. It can be seen that polyphenols released from CS-ALG PECs were decreased with increasing of polyphenols concentration. It was observed that nearly 60% of polyphenols were released from PECTP0.25 in 3 hours, from PECTP0.5 in 4 hours and from PECTP1 in 6 hours, respectively. The accelerated release of polyphenols from chitosan-alginate PEC incubated in high pH media is more likely owed to the reduced electrostatic interaction between the polysaccharide-based polyionic complexes at this pH [27].

The release of polyphenols from CS-ALG PECs incubated in simulated intestinal fluid (pH 7.4) was much faster than the release of polyphenols from polyelectrolytes incubated in simulated gastric fluid (pH1.2), this property is significant for the protection of polyphenol loss in an acid environment.

Within the first three hours the release of polyphenols at pH 1.2 varied from 17.8-29.6 % (PECTP0.25-PECTP1). Thus, can be observed that the polyphenols release from alginate-chitosan polyelectrolytes is pH sensitive and more polyphenols were released in the intestinal system (pH 7.4) than in the gastric system (pH 1.2). Furthermore gastric protection against polyphenols release can be attributed to the most effective retention by a tight ALG network that forms at low pH [28].

Release kinetics

The curve fitting and plotting was performed in Excel. Linearization of polyphenols release profiles at pH 7.4, using the equations (2), was used to characterize the differences found among obtained PECs. The correlation coefficient (r^2) was used as an indication of the best fit. The linearity found *in vitro* drug release of polyphenols by

the Korsmeyer-Peppas model was r^2 = 0.976 for PECTP0.25, r^2 = 0.988 for PECTP0.5 and r^2 = 0.981 for PECTP1. The exponent *n* in Korsmeyer-Peppas equation indicate the type of the release mechanism, thus, values below 0.43 correspond to a Fickian diffusion mechanism and values of *n* greater than 0.85 indicated super case II transport. The values of *n* between 0.43 and 0.85 could be regarded as indicators of both phenomena (transport corresponding to coupled drug diffusion in the hydrated matrix and polymer relaxation), commonly called anomalous non-Fickian transport [29]. In our study, the values of *n* ranged between 0.76 and 0.83 which appeared to indicate a coupling of the diffusion and polymer relaxation mechanisms, and might show that the polyphenols release from PECs could be controlled by more than one process.

4. Conclusions

Alginate-chitosan polyelectrolytes complexes were prepared for the entrapment and controlled release of natural polyphenols. DSC and FTIR confirmed the existence of interactions between alginate/chitosan /polyphenols. It was observed that for a mass ratio range alginate: chitosan 30: 3.2 (w/w) the particle size of PECs varied, from 1261 to 2384 nm, with polyphenols: alginate mass ratio 0.53, 0.26, 0.13 (w/w). The smallest particles were obtained by the decreasing of pH from 5.2 to 4.7 when slightly decreased the mean particle size of obtained PEC from 1261 nm to 962 nm. The release results demonstrated that chitosan-alginate PEC has pH–responsive release. Among all PECs the formulation PECTP1 was assessed as beeing the best considering the particle size, entrapped polyphenols content, in vitro release. In conclusion, the prepared alginate-chitosan PEC offers an interesting system which might be efficiently applied for delivery of natural polyphenols.

Further researches should be oriented to the possibility of developing a delivery system where high-value bioactive molecules quantified in miscellaneous natural extracts can be integrated to have a synergistic effect.

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