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ORIGINAL ARTICLE

Chitosan nanoparticles as a new delivery system for the chemotherapy agent tegafur

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Abstract

Background: Despite the very efficient antitumor activity of conventional chemotherapy, generally high doses of anticancer molecules must be administered to obtain the required therapeutic action, simultaneously leading to severe side effects. This is frequently a consequence of the development of multidrug resistance by cancer cells and of the poor pharmacokinetic profile of these agents. *Objective*: In Order to improve the antitumor effect of tegafur and overcome their important drawbacks, we have investigated its incorporation into a drug nanoplatform based on the biodegradable polymer chitosan. *Materials and Methods*: Two tegafur loading methods were studied: (i) absorption into the polymeric network (entrapment procedure); and (ii) surface deposition (adsorption procedure) in already formed chitosan nanoparticles. *Results*: Tegafur entrapment into the polymeric matrix has yielded higher drug loading values and a slower drug release profile, compared to single surface adsorption. The main factores determining the drug loading to chitosan were identified. *Discussion and Conclusion*: Such polymeric colloid present very interesting properties for efficient tegafur delivery to cancer.

Key words: *Antitumor drug; cancer targeting; controlled release; drug carriers; polymeric colloids*

Introduction

Tegafur [ftorafur, 5-fluoro-1-(tetrahydro-2-furyl)uracil] is a chemotherapy agent belonging to the group of nucleoside analogues. This is a very effective drug in the treatment of a wide variety of solid malignancies (including head and neck, breast, and colon cancers) that, as it is usually the case in conventional chemotherapy, suffers from several drawbacks, mainly a nonuniform oral absorption, a short biological half-life (because of a very rapid metabolism by the enzyme uracil reductase or dihydropyrimidine dehydrogenase), and the inability to overcome the development of multidrug resistance by cancer cells. This generates the need to use high doses that simultaneously lead to severe dose-limiting side effects[1,2].

New therapeutic approaches for cancer treatment have been focussed on the design of polymer colloids for the selective delivery of chemotherapy agents into the tumor site, while minimizing their biodistribution. The objective is always to protect the loaded drug from biological metabolization and elimination and to achieve the highest therapeutic effect with minimal toxicity[3,4]. Among the different polymers used in the formulation of these nanosystems, chitosan has gained increased attention for the development of biocompatible, biodegradable, and nontoxic drug carriers. In fact, this natural linear biopolyaminosaccharide has shown great potential for pharmaceutical applications (e.g., vehicle for directly compressed tablets, disintegrant, binder, granulating agent, or drug carrier), because of its good biocompatibility, biodegradability, targetability, high charge density, nontoxicity, and mucoadhesion[5,6]. Chitosan micro- and nanoparticles (NPs) are under extensive investigation both for parenteral and oral delivery of many active agents (from low molecular weight compounds to macromolecular drugs) in order to improve the bioavailability of degradable substances such as proteins or to enhance the uptake of hydrophilic substances across epithelial layers. The in vivo susceptibility

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of chitosan to lysozyme makes it biodegradable and an ideal material to provide controlled release of many drugs, such as anticancer agents, antidiabetics, antihypertensive agents, diuretics, anti-inflammatories, antibiotics, antithrombotics, steroids, peptides, proteins, amino acids, and vaccines[7–9].

Hence, this work will focus on the formulation of tegafur-loaded chitosan NPs. The therapeutic efficiency of this antitumor drug could be significantly enhanced by its incorporation into this polymeric colloid, thanks to (i) a controlled delivery to the targeted site that will enhance its pharmacokinetic profile and (ii) the minimization of the adverse side effects, a consequence of a minimized biodistribution. The parameters that determine the amount of tegafur loaded to the chitosan NPs, both into the polymeric matrix and onto the polymeric surface, were investigated. In particular, the polymer, surfactant, and drug concentration. Finally, the in vitro tegafur release profiles were also characterized according to the drug-loading procedure. Spectrophotometry was validated and used successfully, as the analytical technique in the quantitative determination of both drug-loading and release profiles. The adsorption of this chemotherapy agent onto chitosan NPs was also qualitatively determined by electrophoresis.

Materials and methods

Materials

Water used in the experiments was deionized and filtered with a Milli-Q Academic System (Millipore, Molsheim, France). All chemicals used were of analytical quality from Panreac (Barcelona, Spain), except for tegafur, low molecular weight chitosan, and pluronic® F-68 (Sigma-Aldrich, Seelze, Germany).

Methods

Preparation of chitosan nanoparticles

Chitosan NPs were prepared by a coacervation method. In this synthesis procedure, the addition of sodium sulfate to the solution of chitosan in acetic acid resulted in decreased solubility of chitosan, rapidly leading to its precipitation as a poorly soluble derivative. This process avoids the use of toxic organic solvents and glutaraldehyde, which are widely used in the other methods of preparation of chitosan particles[6,10]. Briefly, chitosan (1%, w/v) was dissolved in 50 mL of an aqueous solution of acetic acid (2%, v/v) containing 1% (w/v) pluronic® F-68. About 12.5 mL of a solution of sodium sulfate (20%, w/v) was added dropwise (2.5 mL/min) to the chitosan solution under mechanical stirring (1200 rpm). After the addition of sodium sulfate, stirring was continued for 1 hour to obtain the aqueous suspension of chitosan NPs. The colloidal suspension was then subjected to a cleaning procedure that included repeated cycles of centrifugation (40 minutes at 73,920 × g, Centrikon T-124 high-speed centrifuge; Kontron, Paris, France) and re-dispersion in water, until the conductivity of the supernatant was ≤ 10 μS/cm.

Drug loading to chitosan NPs was achieved by following two procedures: The first method (entrapment procedure) followed for tegafur absorption into the polymeric NPs was similar to that above described for the preparation of chitosan NPs, except that the aqueous phase was a 2% (w/v) acetic acid and a 1% (w/v) pluronic® F-68 aqueous solution, with appropriate amounts of the antitumor drug. In this method, the influence on drug entrapment of the concentration of polymer and stabilizing agent was also investigated. The second one (adsorption procedure) involved single drug surface adsorption onto the preformed polymeric NPs: a suspension of chitosan NPs (2%, w/v) was incubated for 24 hours (at 25.0 ± 0.5°C, and under mechanical stirring: 50 rpm) with increasing amounts of tegafur (up to 0.01 M).

Characterization methods

Mean particle diameters were determined at 25.0 ± 0.5°C by quasi-elastic light scattering using a Nanosizer (Coulter® N4MD, Coulter Electronics Inc., Hialeah, FL, USA). The scattering angle was set at 90°, and the measurement was made after suitable dilution of the aqueous NP suspensions. Each measurement was performed in triplicate. The stability of the formulations was evaluated by measuring the size of the particles after 2 weeks of storage at 4.0 ± 0.5°C in water. To confirm the size measurements, the chitosan aqueous suspensions were checked through analysis by high-resolution transmission electron microscopy (TEM) and scanning electron microscopy pictures, obtained using STEM PHILIPS CM20 (the Netherlands) high-resolution transmission, Zeiss EM 902 (Oberkochen, Germany) transmission, and Zeiss DSM 950 (Germany) scanning electron microscopes, respectively. Before observation, dilute suspensions [≈0.1% (w/v)] were sonicated for 5 minutes, and drops were placed on copper grids with formvar film. The grids were then dried at 25.0 ± 0.5°C in a convection oven. Chitosan NPs were visualized using TEM after freeze-fracture. This method consisted of three consecutive steps: high-pressure freezing, freeze-fracture, and TEM study of the preparations.

UV absorption measurements were carried out to determine the drug concentration in all the systems investigated: at the maximum absorbance wavelength (271 nm) in a 8500 UV–Vis Dinko spectrophotometer (Dinko, Barcelona, Spain), using quartz cells of 1-cm path length. Good linearity was observed at this wavelength

and the method has been validated and verified for accuracy, precision, and linearity in all conditions tested.

Determination of tegafur loaded to the chitosan nanoparticles

The determination of the amount of drug loaded to chitosan was performed in triplicate by means of spectrophotometric determinations of the drug remaining in the supernatant (after NP centrifugation: 40 minutes at 73,920 × g), which was deduced from the total amount of tegafur in the NP suspension. For the method to be accurate, we considered the contribution to the absorbance of sources other than variations in drug concentration (mainly the surfactant agent) by subtracting the absorbance of the supernatant produced in the same conditions but without the antitumor agent. The most important reason for absorbance changes is the presence of surfactant, unprecipitated polymer, and by-products of the polymer degradation in the medium. This justifies the procedure used to estimate the drug loading[11–14]: the amount of tegafur present in the solution after the synthesis of the NPs was obtained from the absorbance of the solution at a wavelength of 271 nm, after substracting the absorbance of the supernatant produced in the same conditions but without drug in solution. In fact, we validated the method by comparing the evaluation of drug concentration in two instances: a certain amount of drug was dissolved in the supernatants of polymer syntheses (done in the absence of drug), and the same amount was dissolved in equal amounts of water. We found that the concentrations estimated (in the first case from the difference between the absorbance of the drug plus supernatant solution and that of the supernatant) were identical within the experimental uncertainty. These tests were carried out in sextuplicate, and demonstrated the reproducibility of the method and the absence of molecular interactions.

Drug incorporation to NPs was expressed in terms of tegafur loading (%) [(encapsulated drug (mg)/carrier (mg)) × 100] and tegafur entrapment efficiency (%) [(encapsulated drug (mg)/total drug in the colloidal suspension (mg)) × 100][14].

A qualitative follow-up of the adsorption process was done by means of electrophoretic mobility (u_e) determinations of chitosan NPs in dilute suspensions [≈0.1% (w/v)] with different drug concentrations, using a Malvern Zetasizer 2000 (Worcestershire, UK) electrophoresis device. Measurements were performed at 25.0 ± 0.5°C, after 24 hours of contact at this temperature under mechanical stirring (50 rpm). The experimental uncertainty of the measurements was <5%. To evaluate the effect of ionic strength variations, we performed the experiments both with and without 1 mM KNO_3 in solution.

In vitro release studies of tegafur from chitosan nanoparticles

The study of drug release from chitosan NPs loaded with tegafur after a single adsorption process or after drug incorporation into the polymeric matrix was undertaken with the NPs prepared following the best drug-loading conditions: a 10^{-2}-M tegafur concentration in the adsorption/absorption process.

Drug release from NPs was performed in vitro following the dialysis bag method, and using phosphate-buffered saline (pH 7.4 ± 0.1) as the release medium. The bags were soaked in water at 25.0 ± 0.5°C for 12 hours before use. The dialysis bag (cutoff of 2000 Da, Spectrum® Spectra/Por® 6 dialysis membrane tubing, USA) retained the NPs, but allowed the free drug to diffuse into the dissolution medium. Two milliliters of NP suspension (containing 3 mg/mL of tegafur) was placed into the dialysis bag with the two ends fixed by clamps. The bags were placed in a glass beaker containing 100 mL of the dissolution medium and stirred at 200 rpm. The temperature was maintained at 37.0 ± 0.5°C during these experiments, which were performed in triplicate. At prefixed time intervals (0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 9, 24, 48, 72, 96, 120, and 144 hours), 5 mL of the medium was withdrawn and analyzed for the drug content using UV–Vis spectrophotometry (at 271 nm). An equal volume of phosphate-buffered saline, maintained at the same temperature, was added after sample withdrawal to ensure the sink conditions. The same analytical procedure used for the quantification of tegafur loading was followed in this study.

Results and discussion

Particle size and morphology

The coacervation method followed for the synthesis of chitosan NPs allowed the formation of well-stabilized spherical nanospheres consisting of a well-defined matrix[6,10], with an average diameter of 160 ± 25 nm and a narrow size distribution (polydispersity index: 0.056) (Figure 1). This figure includes a TEM picture taken after freeze-fracture and an SEM image that clearly show that chitosan forms a matrix (nanosphere). No presence of aggregates or bulky sediments was observed. The size of chitosan NPs and the quality of the suspensions did not vary significantly when loaded with different amounts of tegafur. In addition, no drug precipitation or NP aggregation was observed, and no appreciable change in the size of NPs was detected by quasi-elastic light scattering after 2 weeks of storage at 4.0 ± 0.5°C in water.

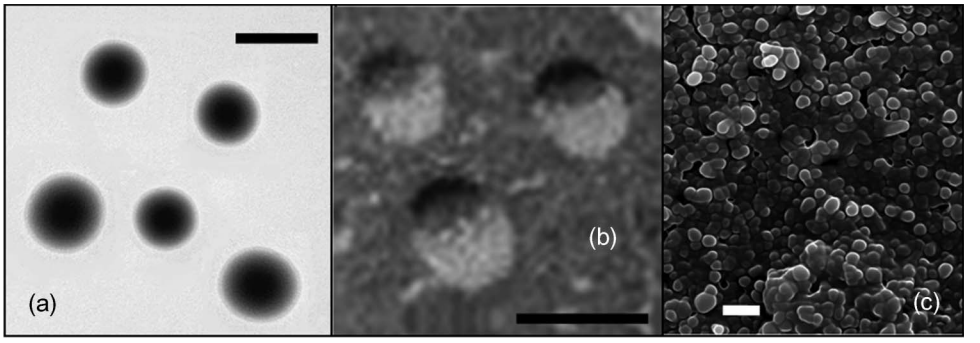

Figure 1. High-resolution transmission electron microscope photograph (a), transmission electron microphotograph after freeze-fracture (b), and scanning electron picture (c) of chitosan nanoparticles. Bar lengths: 200 nm (a, b) and 500 nm (c).

Tegafur surface adsorption onto chitosan nanoparticles

Figure 2 shows the amount of antitumor drug adsorbed at the surface of chitosan as a function of the equilibrium drug concentration. As can be observed, tegafur adsorption efficiency onto chitosan NPs was positively influenced by the drug concentration in the incubation medium: an increase in tegafur concentration leads to larger adsorbed amounts, although a sort of saturation is suggested at high equilibrium concentrations. The entrapment efficiency (%) of tegafur to chitosan increased with the amount of drug in solution up to ≈9.2% for the range of concentrations investigated (Figure 2a). Thus, when using the surface adsorption procedure, the drug loading can be considered very low (maximum drug loading ≈0.9%; Figure 2b). These results could be explained if we consider that the drug must be positively charged in the aqueous medium (presumably because of the protonation of the –NH group of the drug molecule)[15], and unfavorable electrostatic interactions will exist with the positively charged chitosan NPs[16], thus leading to low tegafur surface adsorption. Only the presence on the polymer backbone of hydrophobic units (acetamide groups) could slightly contribute to the surface adsorption of this lipophilic drug (water solubility of tegafur ≈8 mg/mL at room temperature)[16,17].

Electrophoretic mobility (u_e) measurements qualitatively confirmed the conclusions based on adsorption determinations. Electrophoresis is very sensitive to surface changes through adsorption of, principally, charged entities, even at rather small amounts. Thus, electrophoretic mobility (u_e) determinations can be used to qualitatively confirm drug adsorption and even suggest the possible role of electrostatic interactions in this physicochemical process[13,14]. Data corresponding to chitosan NPs in drug solutions are shown in Figure 3. In this figure, we have included the values of u_e of the polymer in the absence of drug: note that it is positive

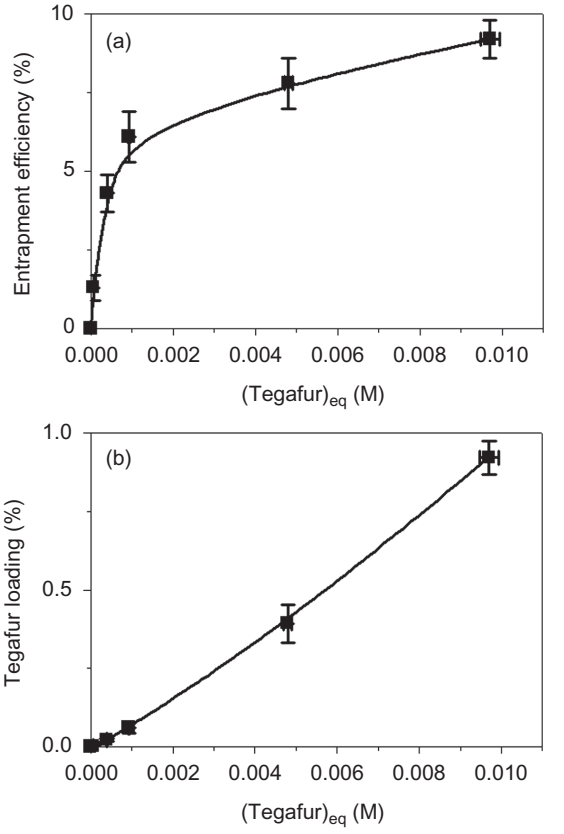

Figure 2. Tegafur entrapment efficiency (%) (a) and tegafur loading (%) (b) values on the surface of chitosan nanoparticles, as a function of the equilibrium drug concentration. The lines are guides to the eye.

for both 1 mM KNO_3 concentration and pure water. As observed, u_e displayed a very small trend to rise toward progressively more positive values as the concentration of tegafur was increased. An unfavorable electrostatic interaction in the aqueous medium between the positively charged chitosan NPs and tegafur molecules

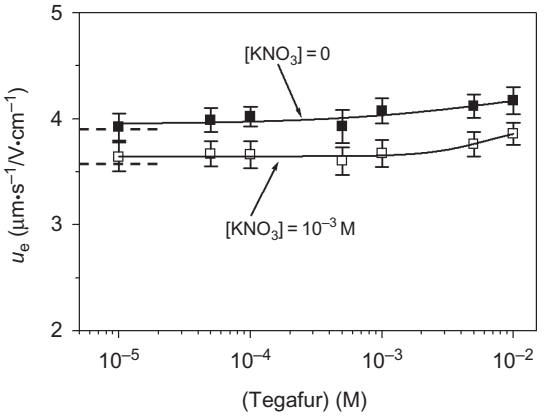

Figure 3. Electrophoretic mobility (u_e) of chitosan nanoparticles (■, □) as a function of tegafur concentration, in the presence (open symbols) or in the absence (full symbols) of 10^{-3} M KNO_3. The dashed lines indicate the mean u_e value of the polymeric nanoparticles in the absence of the drug for 1 mM KNO_3 or in water. The lines are guides to the eye.

can be claimed, pointing out a negligible surface adsorption[15,16]. The originally positive charge is only slightly increased by the very little adsorption of antitumor drug (positively charged species, presumably coming from the protonation of the –NH group of the drug molecule). Finally, the presence of KNO_3 clearly yielded a u_e reduction because of the classical double-layer compression mechanism, in virtue of which the electric potential decreases faster with distance, the larger the indifferent electrolyte concentration. Under these conditions and as a consequence of this small reduction in the chitosan surface charge, KNO_3 could favor to some extent the attraction between the polymer NPs and the positively charged drug molecules. Hence, a slightly more significant enhancement of u_e toward more positive values is observed when tegafur concentration is increased in the presence of KNO_3.

Tegafur absorption into chitosan nanoparticles

Effect of the drug concentration

Because of the previously commented unfavorable electrostatic interaction between tegafur and chitosan, the approximation of this drug from the aqueous phase to the polymeric matrix was supposed to be poor and a great tegafur loading was not expected to take place. However, the preparation conditions tried to reduce the escape of the drug from the chitosan matrix: polymeric NPs precipitated immediately after the beginning of the coacervation method (addition of sodium sulfate to the aqueous solution of chitosan in acetic acid), thus leading to an enhanced mechanical trapping of the drug within the polymer. This strategy could lead to greater tegafur-loading values, as it will be thermodynamically favored: this hydrophobic antitumor drug will keep in contact with the polymer when the NPs are formed rather than as isolated molecules in water. Furthermore, the important role played by the stabilizing agent (pluronic® F-68) has been also well described: it will induce the opening of the polymer chains, thus creating a space within the polymeric network where the drug can be incorporated[18,19].

Figure 4 shows the amount of tegafur absorbed by the chitosan NPs as a function of the equilibrium drug concentration. Compared to the surface adsorption procedure (Figure 2), both the entrapment efficiency (%) and the drug loading (%) were significantly enhanced whatever the initial tegafur concentration fixed. As an example, when the initial drug concentration in the adsorption/absorption medium was 10^{-2} M, these parameters rose from ≈9.2% and ≈0.9% (after tegafur adsorption onto the chitosan surface) to ≈36.9% and ≈18.1% (when tegafur was absorbed into the chitosan matrix), respectively. Furthermore, a positive effect of tegafur concentration on the absorption efficiency into

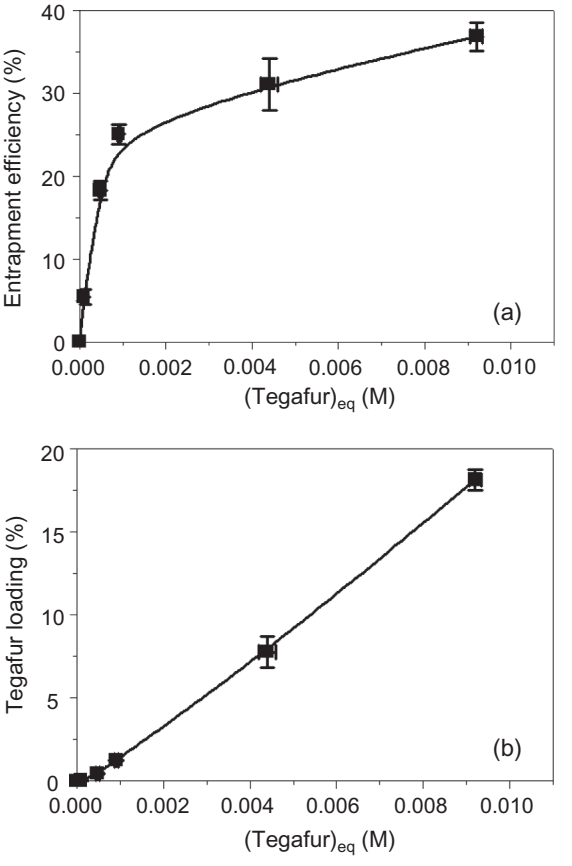

Figure 4. Tegafur entrapment efficiency (%) (a) and tegafur-loading (%) (b) values into chitosan nanoparticles, as a function of the equilibrium drug concentration. The lines are guides to the eye.

the polymeric NPs can be observed, as previously described when following the adsorption procedure.

Influence of the surfactant and polymer concentration

The addition of pluronic® F-68 to the aqueous phase yielded homogeneous distributions of chitosan NPs with reduced size and great uniformity, without influencing the drug incorporation into the polymer[15]. Concerning the effect of chitosan concentration, no significant influence on drug loading was observed when the amount of polymer solubilized in the aqueous phase was enhanced. Only the absolute amounts of tegafur loaded increase when the chitosan concentration rises. On the contrary, the relative or specific drug loading was essentially independent of the amount of polymer added, staying constant at ≈18.1%. Consequently, such an increase in chitosan concentration simply leads to an increment in the formation of tegafur-loaded chitosan NPs[18,20,21].

Release of tegafur from chitosan nanoparticles

The release of tegafur adsorbed onto the chitosan NPs was almost complete within 2 hours, because of a single and rapid desorption process from the chitosan surface (Figure 5). Regarding the release of tegafur absorbed into chitosan NPs, it is apparent from this figure that the in vitro release profile of the drug showed a biphasic process typical of this polymer[22–25], with an initial fast (burst) drug release (up to ≈30% in 1 hour), the remaining chemotherapy agent being released in a sustained manner over a period of 143 hours (≈6 days). The burst release is presumably because of the leakage of the surface-associated and/or poorly entrapped drug, which easily diffuses in the initial incubation time. After the burst release period, the rate of release fell as the dominant release mechanism was changed to drug diffusion through the chitosan matrix[24]. Such a biphasic profile suggests that the major fraction of tegafur was entrapped into the polymeric network rather than adsorbed onto the NP surface. We did not use any statistical model to fit this data, and our conclusion only intended to be a qualitative one. However, we have fitted the data coming from this in vitro release experiment of tegafur from the chitosan NPs to a logistic function with two characteristic points (t_1 and t_2):

$$y = \frac{A_1}{1+\frac{t}{t_1}} + \frac{A_1 - A_2}{1+\frac{t}{t_2}} + A_2$$

and, we found that t_1 and t_2 were ≈1 and 143 hours, respectively.

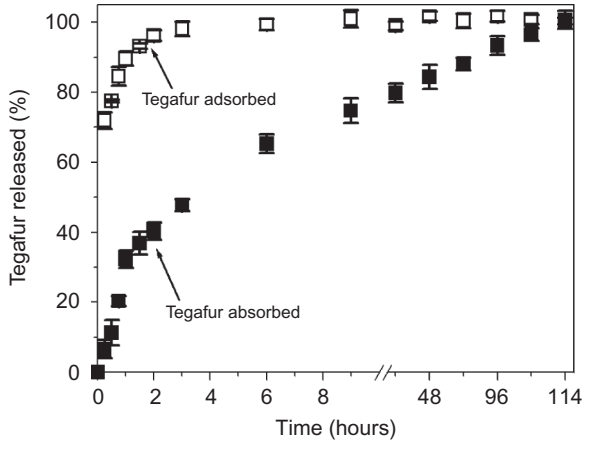

Figure 5. Release of tegafur adsorbed (□) or absorbed (■) from chitosan nanoparticles, as a function of the incubation time in PBS (pH = 7.4 ± 0.1).

Conclusions

The optimal formulation conditions to obtain tegafur-loaded chitosan NPs suitable for parenteral administration were determined. The contributions of both the surface and the polymer network to the overall drug loading were investigated by means of electrophoretic mobility and optical absorbance determinations. Tegafur incorporation into the polymeric matrix during the coacervation process has yielded higher drug-loading values and a sustained drug release profile, compared to single surface adsorption. These very interesting results suggest that chitosan NPs are potential carriers for efficient delivery of tegafur to cancer.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Acknowledgments

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