International Journal of Pharmaceutics 392 (2010) 92–100

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Chitosomes as drug delivery systems for C-phycocyanin: Preparation and characterization

M. Manconi[a], S. Mura[a], M.L. Manca[a], A.M. Fadda[a], M. Dolz[b], M.J. Hernandez[b], A. Casanovas[b], O. Díez-Sales[c,*]

[a] Dpt. Farmaco Chimico Tecnologico, Universita'degli Studi di Cagliari, Italy
[b] Dpt. Física de la Terra i Termodinàmica, Universitat de Valencia, Spain
[c] Dpt. Farmacia y Tecnología Farmacéutica, Universitat de Valencia, Spain

ARTICLE INFO

Article history:
Received 14 December 2009
Accepted 13 March 2010
Available online 23 March 2010

Keywords:
Liposomes coated
Drug delivery
Release study
Diffusion coefficient
Swelling
Mucoadhesive properties

ABSTRACT

The aim of this work was to investigate chitosomes, i.e. liposomes coated by a polyelectrolyte complex between chitosan (CH) and xantan gum (XG), as potential delivery system for oral administration of the protein C-phycocyanin. To this purpose several CH–XG-microcomplexes were prepared in aqueous lactic acid at different chitosan–xanthan gum percent ratios and rheological properties of the microcomplexes were studied to analyse the contribution of chitosan and xanthan gum in the reaction of microcomplexation. After establishing the best microcomplexes, chitosomes were prepared by coating C-phycocyanin loaded liposomes with the CH–XG hydrogels using spray-drying or freeze-drying. The chitosomes were characterized in terms of morphology, size distribution, zeta potential, swelling properties, drug release, and mucoadhesive properties. Rheological studies showed the influence of xanthan gum in the microcomplex properties. Moreover, obtained results demonstrated the effects of formulation and process variables on particle size, drug content, swelling, drug release, and especially on the mucoadhesiveness of C-PC chitosomes of CH–XG. In particular, chitosomes prepared by spray-drying technique using CH–XG in 0.5/8.0 (w/w) ratio showed a regular surface and a drug release characteristic for a Fickian diffusion of the active ingredient. The in vitro mucoadhesive study revealed that the spray-drying method is advantageous to prepare C-phycocyanin loaded chitosomes with excellent mucoadhesive properties for colonic drug delivery.

1. Introduction

C-phycocyanin (C-PC) is one of the main biliprotein constituent of blue-green algae such as Spirula (Arthospira) maxima. In these organisms, they act as accessory pigments for photosynthetic light collection. At present C-PC is used as a nutrient-dense source in food (Othes and Pire, 2001; Pugh et al., 2001), as a colorant in cosmetic and food (Yoshida et al., 1996) and in biomedical applications (Glazer, 1994; Bhat and Madyastha, 2000). In pharmaceutical research, C-phycocyanin has shown radical scavenging properties in oxidative stress-induced diseases (Bhat and Madyastha, 2000; Romay et al., 1998) and it has been described as a strong antioxidant (Romay et al., 1999, 2003; Reddy et al., 2000; Benedetti et al., 2004). Other studies suggest that this natural product could be used in the prevention and treatment of some neurological disorders (Rimbbau et al., 1999) and cancer pathologies (Subhashini et al., 2004). In vivo anti-inflammatory effects in mouse have also been reported (Romay et al., 1999). It has also been described its in vivo capability to prevent acetic acid-induced colitis, following oral administration, in rats (Gonzalez et al., 1999) due to inhibition of cyclooxygenase (COX).

Colloidal carriers have been used to protect drugs and improve their action time. For these purposes, different colloidal drug delivery systems such as liposomes, micelles, nanoemulsions, and nanoparticles have been produced (Barratt, 2003; Cevc, 2004). These systems have shown to improve drug bioavailability, modify pharmacokinetics, and protect the encapsulated drug from enzymatic attack. However, in many cases the application of colloidal drug carriers is limited by their stability in solution and in biological environment. Recently, it has been shown that these carriers may be conveniently protected and stabilized by coating them with different polymers (Iwanaga et al., 2000; Xu et al., 2007a; Thirawong et al., 2008). Indeed, researchers of polymer chemistry have developed a wide range of very powerful sophisticated polymers to solve specific medical problems.

* Corresponding author at: Department of Pharmaceutics and Pharmaceutical Technology, University of Valencia, Avd Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain. Tel.: +34 963543321; fax: +34 963644911.
E-mail address: octavio.diez@uv.es (O. Díez-Sales).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.03.038

On the other hand, delayed drug release induced by bioadhesive polymers could lead to increased oral bioavailability of a drug. Mucoadhesive dosage forms have been used to increase drug retention time and to improve the absorption of poorly absorbable drugs because of their ability to adhere to the mucus layer (Xu et al., 2007a,b). Chitosan has been shown to interact with mucin and its hydrated form also shows good mucoadhesive properties (Fiebrig et al., 1995; Hejazi and Amiji, 2003). Liposomes coated with chitosan have also shown to have a prolonged residence time in the gastrointestinal (GI) tract of rats in comparison to uncoated liposomes (Takeuchi et al., 1996). Complexes with a chitosan gel core and a polycation–polyanion membrane have been widely investigated for colon-specific delivery because they exhibited excellent mucoadhesive properties (Wittaya-areekul et al., 2006). The use of biodegradable polymers as carriers for drug delivery has gained a wide interest, mainly for their biocompatibility and for their ability to provide local as well as temporal controlled release of the drug (Hejazi and Amiji, 2003; Mladenovska et al., 2007; Xu et al., 2007a).

In the present work, in an attempt to enhance and control C-PC delivery to intestinal tract, C-PC-encapsulated liposomes were coated with chitosan–xanthan (chitosomes) to develop a carrier capable of preserving the protein stability in the gastrointestinal tract and enhancing the residence time of drug in the colon tract thus improving its efficacy.

2. Material and methods

2.1. Materials

Soy lecithin (SL) was purchased from Galeno (Prato, Italy). Xanthan gum (XG) molecular weight (approximately 3×10^5 g/mol) (batch 035k0199) and chitosan (CH) with average molecular weight of 7.5×10^5 g/mol (75–85% deacetylated) (batch 01518AD), C-phycocyanin (C-PC), cholesterol (Chol) and all the other products were analytical grade and were purchased from Sigma–Aldrich, Milan, Italy. Phosphate buffer solution (PBS) pH 7 was obtained from Carlo Erba Reagents (Rodano, Italy).

2.2. Vesicle preparation

Multilamellar vesicles (MLVs) were prepared according to the thin film hydration method. SL and Chol in chloroform solution were mixed in an equimolar ratio. The lipid mixture was deposited as a thin film by roto-evaporating the chloroform under vacuum. The film was hydrated with C-PC solution (20 mg/mL) and dextrose 1 mM in phosphate buffered saline solution (PBS, pH 7.0) at room temperature by mechanical shaking for 2 h. The final lipid concentration was SL 30 mg/mL, Chol 15 mg/mL in all cases.

2.3. Chitosome preparation

6.5 g of chitosan was dissolved in lactic acid aqueous solution and mixed homogeneously. Distilled water was added up to 1 L and pH adjusted to 5.6 by addition of NaOH (0.1 mol/L) solution. Xanthan solution was obtained by dissolving 6.5 g of xanthan in 1 L of distilled water and mixing homogeneously. C-PC liposomes were added to chitosan gel (chitosomes). Homogeneous complex of two polymers was formed mixing liposomes and chitosan solution with xanthan (50 mL of liposomes in l L of final microcomplex of the two polymers), at different weight ratio under stirring at 25 °C for 20 min. We prepared chitosomes (CH–XG) containing from 0.5% (w/w) of chitosan and xanthan gum ranging from 2% to 10% (w/w). Vesicles were then spray-dried (SD) or freeze-dried (FD). Spray-drying was performed using a Minispray Dryer (Büchi 190, Switzerland) with a standard 0.7 mm nozzle. The inlet temperature, spray flow and compressed spray air flow (represented as the volume of the air input) were set at 140 °C, 6 mL/min and 10 mL/min, respectively. Otherwise, samples were frozen at −20 °C and freeze-dried overnight, using a Criotecnica freeze-drier apparatus (MM Cota Company, Roma, Italy) with 60 mmHg operative pressure at −80 °C.

2.4. Preparation of the tablets

Tablets with a total weight of 350 mg (C-PC ≈ 35 mg) and 9 mm diameter were compressed on an eccentric press (Korsch Type EKO, Frankfurt, Germany) at a compression force of 5–6 kg/cm^2. The tablet strength was determined by a Pharma Test strength tester (PTB311, Pharma Test, Hainburg, Germany) and the friability by a friabilator (Erweka GmbH, Frankfurt am Main, Germany).

2.5. Rheological tests

Viscoelastic measurements were carried out using a Haake Rheostress 1 rheometer (Thermo Haake, Germany) with data acquisition software (RheoWin 3.61) and a circulator bath (DC 30) for temperature control. A cone-plate of 2° and 35 mm diameter was used. All measurements were made in triplicate at 25 °C. The microparticles (200 mg) was slightly agitated in distilled water (1800 mg) and left overnight before measurement. Samples (gel) were allowed to rest for at least 300 s prior to analysis. In all cases, the exposed edges of the sample were covered with silicone oil (Dimethicone, RFE/Ph. Eur.) to prevent evaporation of water during measurement. In order to determine the linear viscoelastic range, stress sweeps at a frequency of 1 Hz were performed for all systems studied.

Frequency sweep tests were performed from 0.01 Hz to 10 Hz, at 1 Pa for all CH–XG systems. The storage modulus (G'), the loss modulus (G'') and loss tangent ($\tan\delta = G''/G'$) were the oscillatory parameters used to compare the viscoelastic properties for all the systems.

For creep and recovery tests, a constant stress in the linear region (1 Pa) was applied instantly and maintained for a period of 300 s (creep) and the compliance (J) was measured. After removing the stress, compliance values were also measured during 300 s (recovery).

The creep data were analysed according to the Burger model (Eq. (1)), consisting of one Maxwell unit and one Kelvin–Voigt unit in series (Diez-Sales et al., 2007).

$$J(t) = \frac{1}{G_0} + \frac{1}{G_1}\left[1 - \exp\left(\frac{-tG_1}{\eta_1}\right)\right] + \frac{t}{\eta_0} \quad (1)$$

where $J(t)$ represents the overall compliance at any time t, G_0 is the instantaneous elastic modulus of the Maxwell unit, and G_1 is the elastic modulus of the Kelvin–Voigt unit. The residual viscosity, η_0, corresponds to the Maxwell element and the internal viscosity, η_1 corresponds to the dashpot associated with Kelvin–Voigt.

The experimental values of compliance J (Pa^{-1}) in the recovery process were fitted to the following empirical equation (Corrias et al., 2008):

$$J(t) = J_\infty + J_{KV}\exp(-Bt^C) \quad (2)$$

where B and C are parameters which define the recovery rate of the system, J_∞ is the residual compliance, J_{KV} is the maximum compliance of the Kelvin–Voigt element, and t is time.

Additionally, Maxwell's spring deformation, or initial shear compliance J_0, was obtained by using Eq. (3), where J_{MAX} is the maximum deformation corresponding to the experimental compliance value for the longest time (300 s) in the creep transient analysis.

$$J_0 = J_{MAX} - (J_\infty + J_{KV}) \quad (3)$$

Finally, from J_{MAX} and J_{∞} values it is possible to obtain the total recovery (R, %) using the following expression:

$$R = \frac{J_{MAX} - J_{\infty}}{J_{MAX}} \times 100 \quad (4)$$

The experimental values were fitted using the KaleidaGraph software (Synergy Software©KaleidaGraph, version 3.51).

2.6. Liposome and chitosome characterization

The liposomes and chitosomes were sized using a Malvern ZetaSizer3 apparatus (model MS 1002, Malvern, UK), determining the volume mean diameter (VMD), polydispersity index (PI) and zeta potential (ZP). VMD and PI were detected by dynamic light backscattering by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The nano-ZS systematically and automatically adapts to the sample by adjusting the intensity of the laser and the attenuator of the photomultiplier, thus ensuring accuracy and reproducibility of the experimental measurement conditions. The polydispersity index (PI) was used as a measurement of the size distribution. PI less than 0.4 indicates a homogenous and monodisperse population. The ZP of the systems was measured as the particle electrophoretic mobility means of laser microelectrophoresis in a thermostated cell by laser Doppler anemometry (Malvern ZetaSizer 3). All the samples for size and zeta potential were analysed immediately and 24 h after their preparation.

The morphology of the chitosomes was evaluated by a Hitachi S4100 scanning electron microscope (SEM) and morphology of liposomes was studied by a Jem1010, Jeol transmission electron microscope (TEM).

Uncoated liposomes and chitosomes were purified from the non-encapsulated C-PC by centrifugation (Mikro 200, Hettich) at 14,000 rpm for 20 min at 4 °C and washed three times with PBS (pH 7.0). Encapsulation efficiencies (E, %) (expressed as a percentage of the total amount of C-PC found in the studied formulations at the end of the preparation procedure) were determined spectrophotometrically after disruption of vesicles. Vesicles were broken with 1% Triton X-100 in PBS (pH 7.0); chitosomes were dissolved in methanol. Fluorimetric methods were used for determination of drug contents. Samples were analysed using a Fluorescence Spectrophotometer Hitachi F2000. C-PC solution was excited at 600 nm and emission of fluorescence was detected at 640 nm.

2.7. Swelling studies

The swelling degree of chitosome tablets was determined by keeping dried test samples in 10 mL of buffered solution (pH 1.5 and 7.4, respectively) at 37 °C for 24 h. At specific time intervals, samples were removed from the swelling medium and blotted with a piece of paper for 5 s to absorb excess water on surface. The swelling ratios (S_{wt}) of the test samples were calculated from the following equation:

$$S_{wt}(\%) = \frac{W_t - W_d}{W_d} \times 100 \quad (5)$$

where W_t is the weight of the swollen test sample and W_d is the weight of the dried test sample. Each W_d of samples was no less than 0.40 g.

2.8. In vitro release study

In vitro release profiles of C-PC from tablets were examined in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) using USP dissolution apparatus. The tablet (equivalent to 10 ± 2 μg of C-PC) was put into the basket and placed in 20 mL of the dissolution medium, thermostated at 37.0 ± 0.5 °C. The drug dissolution was determined in SGF and SIF media for 24 h at 100 rpm. At scheduled time intervals agitation was stopped, the samples (1 mL) were withdrawn and replaced with fresh medium. The samples were diluted in methanol, filtered and the drug content determined spectro-fluorometrically at 600 nm of excitation and at 640 nm of emission of fluorescence. All the experiments were made in triplicate.

The mean release profiles (cumulative drug release up to 60%) were fitting according to the power law equation (Eq. (6)) in order to describe the drug release mechanism.

$$\frac{M_t}{M_{\infty}} = Kt^n \quad (6)$$

where M_t and M_{∞} are the absolute amount of drug released at t and infinite time, respectively; K is a constant reflecting structural and geometric characteristic of the device, and n is the release exponent characterizing the diffusion mechanism. According to the criteria for release kinetics from swellable cylindric systems, release exponent values $n = 0.45$, $0.45 < n < 0.89$ and 0.89 indicate, respectively, Fickian (case I) diffusion, non-Fickian (anomalous) transport, and diffusion and zero-order (case II) transport (Ritger and Peppas, 1987; Siepmann and Peppas, 2001).

2.9. Preparation of GI tissues and mucoadhesive test

Wistar rats (13-week old) have been fasted for 24 h. The fasted conditions were set to minimize the contents in the GI tract, which disturbed the washing process for the following use. The intestine tissues (i.e. duodenum, jejunum, ileum and colon) were excised from rats that were sacrificed. Each section of tissues was then slowly washed with a large amount of normal saline solution. Then, the intestine tissue (duodenum, jejunum, ileum and colon) was immediately used for this study.

Swelling of tablet was simulated using USP dissolution apparatus in the same condition of release study: 45 min in 500 mL of SGF at 200 rpm and after 500 mL of SIF at 100 rpm, thermostated at 37.0 ± 0.5 °C. At scheduled time intervals tablet mucoadhesion studies were done using different part of rat intestine tissue. At 60 min tablet mucoadhesion using duodenum tissue was studied, at 90 min using jejunum, at 120 min using ileum and at 150 min using colon.

The mucoadhesion study was done using a universal tensile tester (Lloyd Instruments, LR 50K model, UK). The stainless steel plate (L-shape) was fitted by one of its side into the upper and lower jaws of the instrument so as the other surfaces of the plates were facing each other. The rat intestine tissue was stuck at the upper plate surface with the glue, while tablet was stuck on the lower plate. PBS, pH 7.4, was used as a medium and 20 μL were spread on the contact surface between tablet and tissue. Then the upper jaw with tissue stuck on the plate was lowered slowly so that it just touched the tablet surface. No external force was applied. The tablet was kept in contact with the tissue for 5 min and then the upper jaw was slowly moved upward at the speed of 10 mm/min. Liposome dispersion without any polymer was used as a control. All the experiments were done in triplicate. The maximum detachment force (F_{MAX}), i.e. the force required for separating the tablet from the tissue surface was obtained directly from NimaST518.vi software (Nima Technology Ltd., Coventry, England) and the total amount of forces involved in the probe withdrawal from the tissue (work of adhesion, W_{ad}) was then calculated from the area under the force versus distance curve. These parameters were used to compare the different formulations tested.

2.10. Statistical analysis of data

Analysis of variance (ANOVA) and Bartlet's test for homogeneity of variance were performed using SPSS version 10.0 for Windows (SPSS Inc., USA). Post hoc testing ($P < 0.05$) of the multiple comparisons was performed by the Scheffe test.

3. Results and discussion

3.1. Rheological analysis

In this part of the study we investigated the rheological properties of different CH–XG complexes with the aim to check the influence of the xanthan gum concentration on the complexation reaction and the methodology used, spray-dried (SD) and freeze-dried (FD), in the formation of the microparticles.

3.1.1. Oscillatory test

Experimental values of mechanical spectra for the different concentrations of CH–XG complex, obtained in the region of linear behaviour, are shown in Fig. 1 (a and b). In the case of FD complexes (CH–XG from 0.5/2.0 to 0.5/10) and SD complexes (CH–XG from 0.5/6.0 to 0.5/10), there was a predominance of the elastic over the viscous behaviour (G' was greater than G''). As can be seen from the Fig. 1(a), in the case of SD formulation, the storage modulus (G') and the loss modulus (G'') increased as XG concentration increased. On the contrary, for FD system the G' and the G'' values decreased as XG concentration increased. However, in both cases the loss tangent ($\tan\delta = G''/G'$) was always lower than 1. This behaviour is typical of three-dimensional networks and indicates the formation of a true gel.

However, SD complexes using CH–XG between 0.5/2.0 and 0.5/4.0 (w/w) showed a loss modulus (G'') greater than the storage modulus (G'). In Fig. 1(a), as an example, only the behaviour of CH–XG (0.5/2.0, SD) is shown. This is a characteristic behaviour of non-structured systems, with a predominance of viscous over elastic properties.

Therefore, the addition at high weight ratio (from 0.5/6.0 to 0.5/10 (w/w)) of a polyanion (such as XG) to a polycation (CH) led to a gelled matrix (Fig. 1(a)). The storage modulus was larger than the loss modulus, and practically independent of frequency. Similar behaviour was obtained with the FD systems in all range of tested concentrations (CH–XG) (Fig. 1(b)). The prevalence of elastic over viscous nature in gelled systems could be considered an advantage for the development of mucoadhesive systems (Oechsner and Keipert, 1999). Therefore all CH–XG preparations with an elastic behaviour would be useful for the colon-specific delivery of C-PC.

To evaluate the influence of XG on the complexes, hydrogels without CH (control) were also prepared. XG hydrogels (from 2% to 10%, w/w) showed values of storage modulus (G') lower than the CH–XG complexes at the same XG concentrations (Fig. 1(c)). Consequently, these results confirm that the presence of CH induces a complementary reinforcement of the mechanical properties of the system, as a consequence of the complex reaction.

3.1.2. Creep and recovery tests

Creep and recovery analyses were carried out to understand the internal structure of all prepared systems that had shown an important elastic behaviour, i.e. 0.5/2.0 CH–XG and 0.5/4.0 CH–XG SD systems were not considered as their behaviour is mainly viscous (see Fig. 1(a)). As an example, the time dependence of compliance, J, for XG gel (8%), CH–XG (0.5/8.0 SD) and CH–XG (0.5/2.0, FD) is shown in Fig. 2. As it can be observed, the system formulated without chitosan is the more deformable while the CH–XG systems (SD and FD) showed a much smaller deformation thus indicating they are more structured systems.

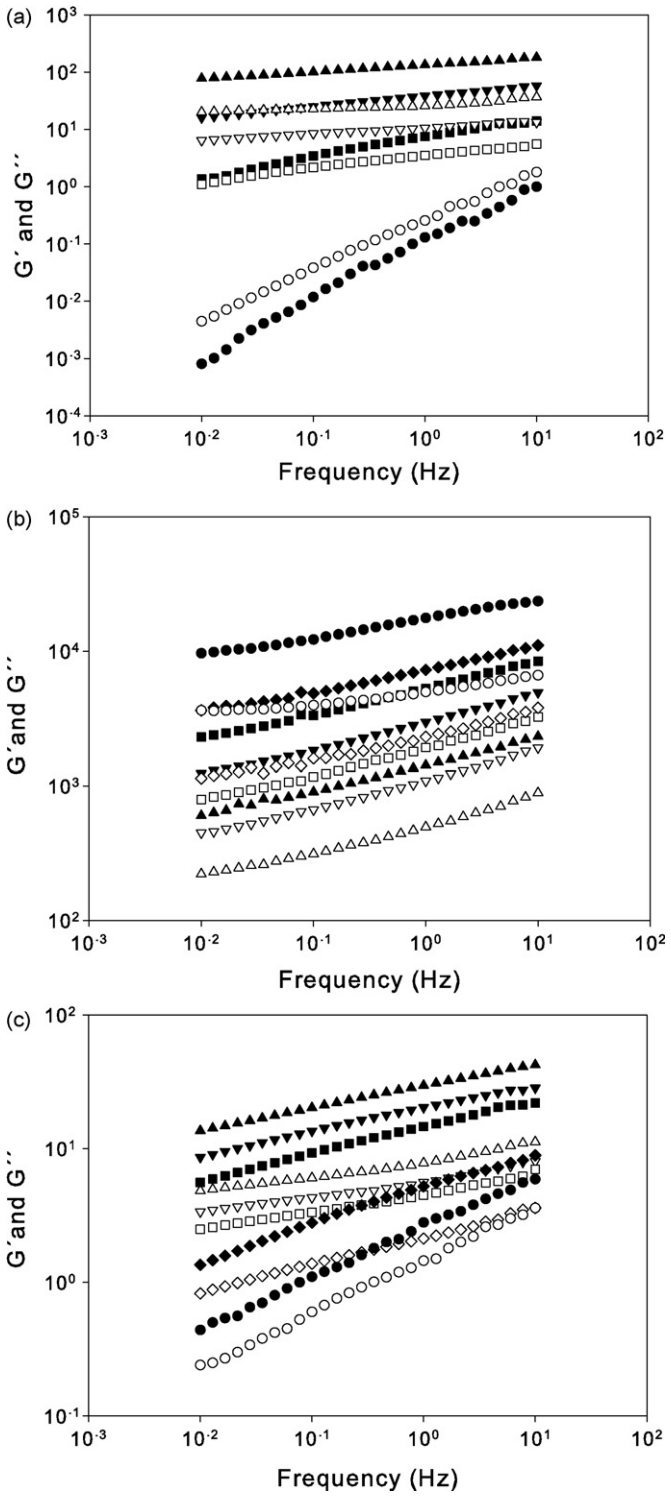

Fig. 1. Magnitude of the storage modulus G', and loss modulus G'' as a function of the angular frequency ω. G' (filled symbols) and G'' (open symbols). (a) Spray-dried (SD) CH–XG complexes; (b) freeze-dried (FD) CH–XG complexes; (c) XG hydrogels at different % (w/w) concentrations: (a) 0.5/2.0 (●,○); 0.5/6.0 (■,□); 0.5/8.0 (▼,□); 0.5/10 (▲,△); (b) 0.5/2.0 (●,○), 0.5/4.0 (♦,◊), 0.5/6.0 (■,□), 0.5/8.0 (▼,▽), 0.5/10 (▲,△) and (c) XG 2.0 (●,○), XG 4.0 (♦,◊), XG 6.0 (■,□); XG 8.0 (▼,▽); XG 10.0 (▲,△).

All the creep curves were fitted to Burger's model (Eq. (1)). The values of the elastic moduli, G_0 and G_1, and the dashpot viscosities, η_0 and η_1, are shown in Table 1. As it is logical, all the parameter values increase when increasing gum concentration in xanthan gum dispersions (without chitosan). Influence of chitosan depends

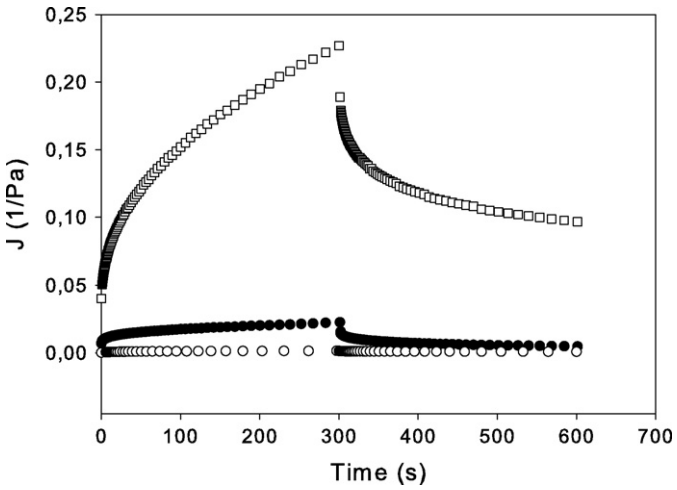

Fig. 2. Creep and recovery compliance curves of XG gel (8%, w/w) (□), CH–XG (0.5/8.0 SD) (●) and CH–XG (0.5/2.0 FD) (O) systems.

on preparation method. For FD method all the parameter values are much higher than those corresponding to the systems withouth chitosan, and they decrease when increasing xanthan gum concentration. However, for SD method, all the parameters increase when increasing xanthan gum concentration and their values are lower than those corresponding to FD method. On the other hand, when comparing with systems without chitosan, we must point out that only system (CH–XG SD) with more than 8% of xanthan gum concentration present higher values of the parameters. In our opinion, the high values obtained when adding chitosan using SD method could be the result of a complexation reaction between xanthan gum and chitosan that is only produced for the highest concentrations due to the different molecular weights of both polymers. The opposite effect in FD method (reduction of parameter values) could be a consequence of frozen-dried effect in formulation procedure.

The compliance values (J_{MAX}, J_0, J_{KV} and J_∞) and total percentage of recovery (R, %) obtained for each element of the Burger model by means of Eqs. (2)–(4) are shown in Table 2. In accordance with creep results (Table 2), systems without chitosan and CH–XG (SD) systems showed a reduction of the compliance values (J, Pa^{-1}) as the XG concentration increased, while FD systems, J values increased with xanthan gum concentration.

Regarding R, % values, it is observed than xanthan gum concentration does not affect significantly systems without chitosan and CH–XG (FD) as the recovery percentage is quite similar. On the other hand, the presence of chitosan implies a lower recovery of the systems. However, in SD systems, xanthan gum concentration clearly affects R, %, reaching even values of about 90%, what is a consequence of the increased contribution of the Maxwell spring deformation (J_0/J_{MAX}, %). Therefore, the FD preparation method provokes a structure that is easier to deform and less reversible.

Moreover, creep and recovery tests allowed us to calculate the gel strength (S) and the relaxation exponent (n), using the Eq. (7)

Table 1
Elastic moduli (G_0, G_1) and dashpot viscosities (η_0 and η_1), using the mechanical Burger's model for xanthan without ($R > 0.997$) and with chitosan microparticles ($R > 0.998$) (CH–XG) obtained by means of the spray-dried (SD) and freeze-dried (FD) methods.

XG conc (%)	G_0 (Pa)	G_1 (Pa)	η_0 (Pa s)	η_1 (Pa s)
	Without chitosan			
2.0	0.98 ± 0.03	0.39 ± 0.01	31.6 ± 0.5	5.6 ± 0.3
4.0	1.16 ± 0.03	0.49 ± 0.01	47.9 ± 0.8	6.7 ± 0.5
6.0	9.5 ± 0.2	6.4 ± 0.1	859 ± 18	166 ± 1
8.0	12.9 ± 0.6	9.8 ± 0.2	1370 ± 30	248 ± 18
10.0	19.3 ± 0.2	16.4 ± 0.4	2480 ± 50	410 ± 30
	With chitosan (FD)			
2.0	2990 ± 70	1570 ± 30	$(5.3 \pm 0.8) \times 10^5$	$(3.20 \pm 0.02) \times 10^4$
4.0	1860 ± 30	878 ± 14	$(3.0 \pm 0.1) \times 10^5$	$(1.25 \pm 0.01) \times 10^4$
6.0	1630 ± 20	820 ± 13	$(2.8 \pm 0.1) \times 10^5$	$(1.21 \pm 0.03) \times 10^4$
8.0	900 ± 12	523 ± 8	$(1.76 \pm 0.06) \times 10^5$	$(0.76 \pm 0.04) \times 10^4$
10.0	690 ± 20	374 ± 8	$(1.06 \pm 0.04) \times 10^5$	$(0.71 \pm 0.01) \times 10^4$
	With chitosan (SD)			
6.0	4.1 ± 0.1	1.83 ± 0.04	105 ± 9	42 ± 2
8.0	30.9 ± 0.3	23.8 ± 0.2	3900 ± 170	540 ± 30
10.0	109.4 ± 2.1	170 ± 10	38,200 ± 2100	3810 ± 140

Results are the mean ± standard deviations ($n = 3$).

Table 2
Compliance values (J_{MAX}, J_∞, J_{KV} and J_0) and total recovery percentage [R, %] for xanthan (XG) without and with chitosan (CH–XG) formulations obtained by means of the spray-dried (SD) and freeze-dried (FD) methods. Results are the mean ± standard deviations in brackets ($n = 3$).

XG (%)	J_{MAX} (Pa^{-1})	J_0 (Pa^{-1})	J_{KV} (Pa^{-1})	J_∞ (Pa^{-1})	R (%)
	Without chitosan				
2.0	12.59 ± 0.04	0.41 ± 0.01	5.94 ± 0.01	6.15 ± 0.01	51
4.0	8.77 ± 0.03	0.26 ± 0.01	5.74 ± 0.04	2.77 ± 0.03	68
6.0	0.576 ± 0.003	0.062 ± 0.003	0.304 ± 0.001	0.222 ± 0.001	64
8.0	0.390 ± 0.002	0.048 ± 0.002	0.214 ± 0.001	0.128 ± 0.001	67
10.0	0.236 ± 0.002	0.038 ± 0.002	0.127 ± 0.001	0.0707 ± 0.0002	70
	With chitosan (FD)				
2.0	$(1.5 \pm 0.1) \times 10^3$	$(0.13 \pm 0.01) \times 10^3$	$(0.82 \pm 0.02) \times 10^3$	$(0.542 \pm 0.002) \times 10^3$	64
4.0	$(2.5 \pm 0.2) \times 10^3$	$(0.17 \pm 0.03) \times 10^3$	$(1.10 \pm 0.01) \times 10^3$	$(1.29 \pm 0.01) \times 10^3$	49
6.0	$(2.7 \pm 0.4) \times 10^3$	$(0.21 \pm 0.06) \times 10^3$	$(1.17 \pm 0.01) \times 10^3$	$(1.32 \pm 0.02) \times 10^3$	51
8.0	$(4.5 \pm 0.5) \times 10^3$	$(0.43 \pm 0.05) \times 10^3$	$(2.35 \pm 0.01) \times 10^3$	$(1.73 \pm 0.01) \times 10^3$	52
10.0	$(6.7 \pm 0.7) \times 10^3$	$(0.61 \pm 0.08) \times 10^3$	$(3.29 \pm 0.02) \times 10^3$	$(2.78 \pm 0.01) \times 10^3$	58
	With chitosan (SD)				
6.0	3.591 ± 0.004	0.147 ± 0.005	1.454 ± 0.009	2.085 ± 0.006	42
8.0	0.146 ± 0.005	0.029 ± 0.006	0.085 ± 0.001	0.039 ± 0.001	73
10.0	0.023 ± 0.002	0.0069 ± 0.00001	0.0137 ± 0.0003	0.0025 ± 0.0002	89

(Nijenhuis, 1997):

$$G(t) = St^{-n} \quad (7)$$

where $G(t)$ is the relaxation function, S is the gel strength parameter, which depends on the cross-linking density and the molecular chain flexibility, and n is related to the molecular structure and connectivity of the incipient gel (Nyström et al., 1996). Exponent n characterizes the critical gel: at $n < 0.5$, the cross-linker is in excess and the opposite holds when $n > 0.5$. $G(t)$ is the relaxation function, which is established from creep test data as the reciprocal of $J(t)$.

Table 3 shows the obtained S and n values. When SD method was used, the complexes strength increased as the concentration of XG increased leading to the most structured, firm, and stable systems obtained with the highest XG concentrations: i.e. SD complexes (CH–XG from 0.5/8.0 to 0.5/10 (w/w)). However, when the FD method was used, the most structured systems were those obtained with the lowest XG concentration (i.e. CH/XG, 0.5/2.0).

On the other hand, Table 3 also shows exponent n value, which is related to the molecular structure of the gel. A high n value can be associated to a spongy gel network while low n values indicate tight gels. The presence of chitosan, in both methods, produces tighter gels. This effect is more important as xanthan gum concentration increase in SD method.

Taking into account all the rheological results obtained, SD complex containing CH–XG 0.5/8.0 (w/w) and FD complex containing CH–XG 0.5/2.0 (w/w) were chosen for C-PC delivery.

Table 3
Results of the gel strength parameter (S), n and correlation coefficients (R) for systems without chitosan and with chitosan and xanthan gum (CH–XG) obtained by means of the spray-dried (SD) and freeze-dried (FD) methods.

XG (%)	S (Pa s^n)	n	R>
Without chitosan			
2	0.87 ± 0.02	0.38 ± 0.01	0.995
4	1.03 ± 0.02	0.36 ± 0.01	0.993
6	10.50 ± 0.20	0.31 ± 0.02	0.993
8	15.03 ± 0.16	0.29 ± 0.03	0.993
10	22.30 ± 0.30	0.28 ± 0.03	0.993
With chitosan (FD)			
2	3130 ± 50	0.28 ± 0.01	0.991
4	1350 ± 20	0.22 ± 0.01	0.993
6	1120 ± 20	0.19 ± 0.02	0.994
8	827 ± 7	0.24 ± 0.01	0.995
10	560 ± 20	0.21 ± 0.01	0.996
With chitosan (SD)			
6	5.17 ± 0.12	0.48 ± 0.04	0.992
8	34.7 ± 0.5	0.27 ± 0.03	0.994
10	119.1 ± 1.8	0.16 ± 0.01	0.996

Results are the mean ± standard deviations ($n = 3$).

3.2. Liposome and chitosomes characterization

Liposomes were prepared using a fixed amount of C-PC (20 mg/mL). To prepare stable vesicles it was necessary to use an amount of 30 mg/mL of SL and 15 mg/mL of Chol. C-PC-liposomes were coated with CH–XG system. Chitosomes containing CH–XG 0.5/8.0 (w/w) were dried by SD method (0.5/8.0 SD) and these containing CH–XG 0.5/2.0 (w/w) were dried by FD method (0.5/2.0 FD). The liposomes coated by FD procedure had an irregular shape and a rough surface, as shown in Fig. 3(a and b). In particular, liposomes coated by CH–XG 0.5/2.0 FD were irregular with aggregated particles forming filaments (A and B). Chitosomes 0.5/8.0 SD joined themselves without fusion and showed a more regular surface (C and D).

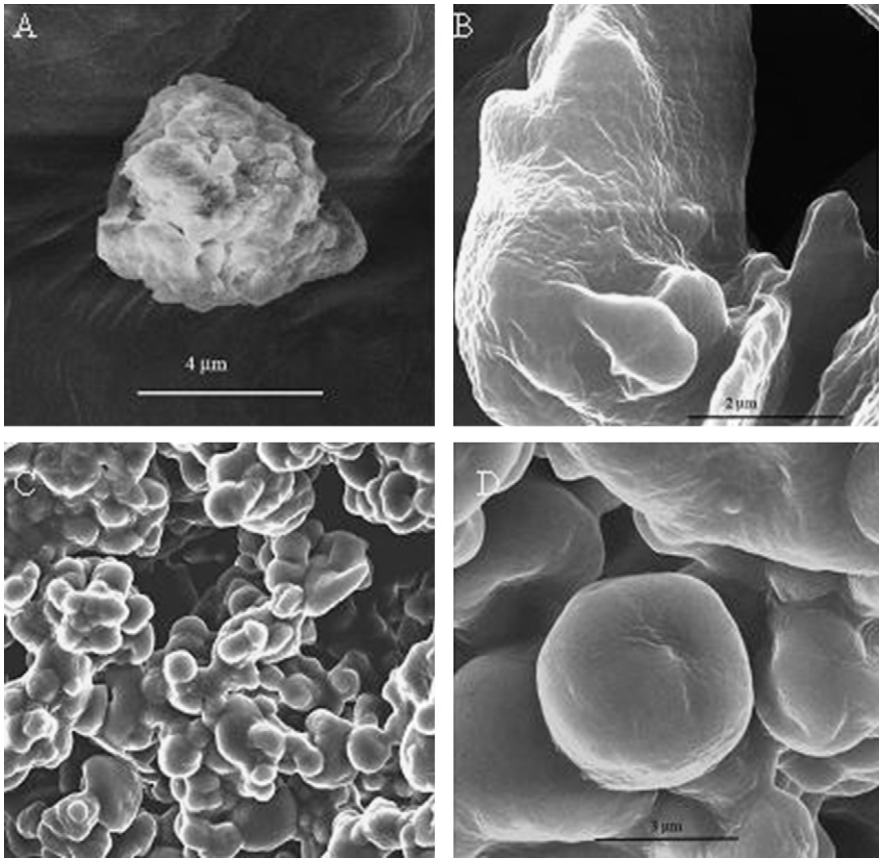

Fig. 3. SEM pictures of chitosomes: FD CH–XG 0.5/2.0 (A and B) and SD CH–XG 0.5/8.0 (C and D).

Table 4
Physico-chemical properties of C-PC-entrapping liposomes and coated liposomes: average size and polydispersity index (PI), *Z*-potential, and encapsulation efficiency (*E*, %).

Formulations	CH/XG coated liposomes			
C-PC liposomes	Size (±SD) μm	PI	Zeta potential (±SD) (mV)	*E* (±SD) (%)
Uncoated	0.416 ± 0.042	0.243	−63 ± 9	30 ± 11
0.5/8.0 SD	3.785 ± 0.376	0.334	−35 ± 7	68 ± 15
0.5/2.0 FD	6.561 ± 0.411	0.427	−26 ± 8	67 ± 13

Results are the mean ± standard deviations ($n = 3$).

As it can be seen in Table 4, C-PC loaded liposomes showed a mean size of 416 nm and a high negative zeta potential probably due to the negative charge of phosphatidylcholine group at pH 7. Table 4 also shows that when the negative-charged liposomes were coated with the CH–XG-microcomplexes a modification of the zeta potential (from −60 to −35 and −26) occurred thus confirming the surface coating. It can also be seen that the CH–XG coating significantly increased the particle size, which ranged from 3 μm to 7 μm.

On the other hand, as it can be seen the encapsulation efficacy increase in the coated systems (Table 4). This fact could be attributed to the partial coalition of the liposomes during the coating process.

3.3. Swelling studies

Swelling degree is a characteristic of hydrogels that controls drug loading as well as drug release. The chitosome tablets reached the highest swelling degree within a period of 24 h (Fig. 4); after that, polymers dissolved. As can be seen, the swelling rate was more rapid for 0.5/2.0 FD formulation than for 0.5/8.0 SD system. For 0.5/8.0 SD samples the swelling profile in SGF and in SIF did not show any appreciable differences. In the case of 0.5/2.0 FD samples, the swelling profiles were similar in both media until the 5th h but afterward the swelling rate increased faster in the acidic medium. The differences observed between chitosomes (0.5/8.0 SD) and chitosomes (0.5/2.0 FD) swelling behaviour may be the consequence of the higher XG concentration in the SD sample and also as a consequence of the different preparation method. In fact, the SD chitosomes were more compact than the FD chitosomes that formed a microporose hydrogel able to swell more rapidly.

3.4. In vitro release study

The in vitro release profiles of C-phycocyanin from the tablets prepared with the chitosomes 0.5/2.0 FD and CH–XG 0.5/8.0 SD were investigated in simulated gastric (SGF, pH 1.2) and intestinal fluid (SIF, pH 7.4) using USP dissolution apparatus. Both types of tablets showed a slower release rate in the acidic medium (Fig. 5). This is probably due to the intramolecular and intermolecular hydrogelation properties of the CH–XG system (Martínez-Ruvalcaba et al., 2007). In SIF, dissolution of the chitosomes promoted the drug diffusion to the external medium. The release of the drug was faster for 0.5/2.0 FD tablets. In fact, as can be seen from the Fig. 5, after 4 h the amount of C-PC released from the FD tablets in SGF and SIF was 49% and 68%, respectively. These results are in agreement with the swelling behaviour of the tablets and they are the consequence of various factors affecting the chitosome structure. In fact, composition of the studied chitosomes is different for the CH–XG ratio in the complex: the highest XG content of the SD product (CH–XG 0.5/8.0 SD) led to a more compact structure able to better control the drug release rate. Moreover, the obtained results are also affected by the different preparation method (i.e. freeze-drying or spray-drying). Therefore, the biggest porosity in the systems elaborated by means of FD method could justify a bigger release rate.

The release data were analysed by applying the power law Eq. (5). The fitting results are presented in Table 5. As can be seen from the obtained correlations coefficient values ($R \geq 0.99$), the release data fit well to the empirical Eq. (5). The *n* values for all formulations are close to 0.45 (from 0.40 to 0.46). Therefore, the drug release from the studied tablets is characteristic for a Fickian diffusion of active ingredient from a cylindric polymeric delivery system (Ritger and Peppas, 1987).

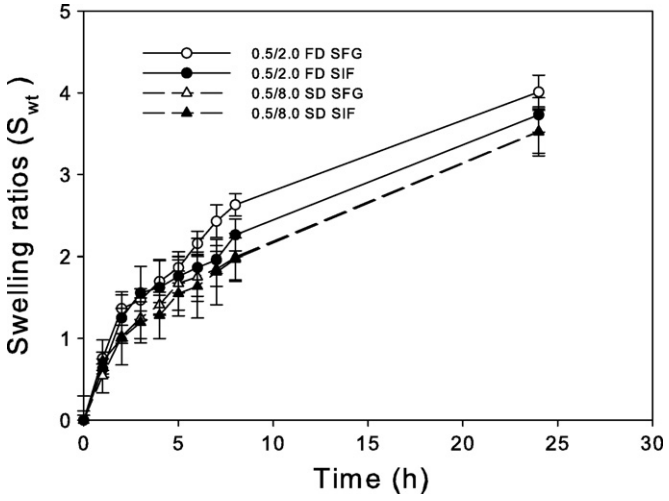

Fig. 4. Swelling characteristics of tablets prepared using spray-dried (SD CH/XG 0.5/8.0) and freeze-dried (FD CH/XG 0.5/2.0) chitosomes in SFG and SIF medium, respectively.

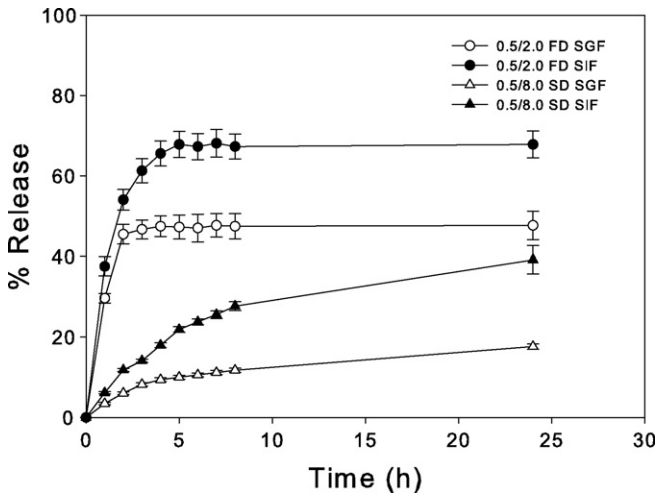

Fig. 5. In vitro phycocyanin release from tablets prepared using spray-dried (SD CH/XG 0.5/8.0) and freeze-dried (FD CH/XG 0.5/2.0) chitosomes, in different test medium ($n = 3$).

Table 5
Comparison of estimate parameters from curve fitting of drug dissolution in pH change media to power law expression.

Formulations	K^a (h^{-1})	n^a	$R>$
0.5/2.0 FD SGF	0.471 ± 0.047	0.40 ± 0.12	0.991
0.5/2.0 FD SIF	0.574 ± 0.023	0.44 ± 0.05	0.998
0.5/8.0 SD SGF	0.073 ± 0.004	0.41 ± 0.03	0.991
0.5/8.0 SD SIF	0.143 ± 0.013	0.46 ± 0.04	0.990

[a] These values are presented with standard errors.

3.5. Ex vivo test for mucoadhesive properties

Gastrointestinal retention depends on many factors such as density, size and resistance of the dosage form, fasting or fed condition, nature of the taken meal, sleep, and posture. It also depends strongly on a complicated and unpredictable gastrointestinal emptying with migrating myoelectric complex motility of the stomach (Talukder and Fassihi, 2004).

To realise colon targeting, modified drug delivery systems with prolonged residence time in the colon and the lower intestine are needed. Adequate control of the intestinal residence time combined with time-controlled drug release patterns can significantly increase drug bioavailability and, thus, the efficiency of the medical treatment. Bioadhesive polymers (chitosan and xanthan gum) could delay drug release in the gastrointestinal tract thus leading to increased oral bioavailability of a drug. It is well known that mucoadhesive systems offer several advantages over other oral controlled release systems since they can extend residence time of drugs in the GI tract, favour localization of the dosage form at a specific site, and reduce local irritation.

In this work, chitosomes were produced also to evaluate the mucoadhesive properties of the obtained systems in comparison with the non-coated liposomes. Therefore, the in vitro mucoadhesive properties of the studied chitosomes (0.5/2.0 FD and 0.5/8.0 SD) were studied using rat's intestine, and the test was carried out on the chitosomal tablets.

The maximum detachment force (F_{MAX}), i.e. the force required for separating the tablet from the tissue surface and the total amount of forces involved in the probe withdrawal from the tissue (work of adhesion, W_{ad}) of both formulations on different GI mucosa are shown in Fig. 6. The figure shows the effect of the contact time between tablets and GI mucosa on the F_{MAX} and W_{ad}. As can be seen (Fig. 6(b)), colon mucosa showed a stronger mucoadhesion than small intestinal mucosa (Schumacher and Schumacher, 1999). These results can be explained considering that the colon does not contain villi but is richer than other GI tracts in goblet cells and therefore it is characterized by high mucin levels that favour mucoadhesion occur easily.

Obtained results also show that the F_{MAX} and W_{ad} have a tendency to increase as the contact time increased in both tablets. Moreover, significant differences between F_{MAX} and W_{ad} values in colon and in small intestinal mucosa ($P<0.05$) were found. This is consistent with values obtained in the literature (Tobyn et al., 1995; Wong et al., 1999) where different types of polymers (e.g. carbomer, polycarbophil, hydroxypropylmethyl cellulose, sodium carboxymethylcellulose) and model mucosa were used. The increase of F_{MAX} and W_{ad} values is most likely due to the degree of hydration and swelling, sufficient to expand the mucoadhesive network. Increasing contact time may provide interdiffusion and chain entanglement between biopolymer and mucin chains in mucus membrane. This is in agreement with Leung and Robinson (Leung and Robinson, 1990), who demonstrated that mucoadhesion of carbomer was a time-dependent process thus supporting the proposed interpenetration as being a time-dependent process. A prolonged contact resulted in an increased formation of secondary bonds and diffusion path or depth of interpenetration between two macromolecules. Increasing contact time between the mucoadhesive polymer and the mucus layer could, therefore, increase the mucoadhesive strength (Leung and Robinson, 1990).

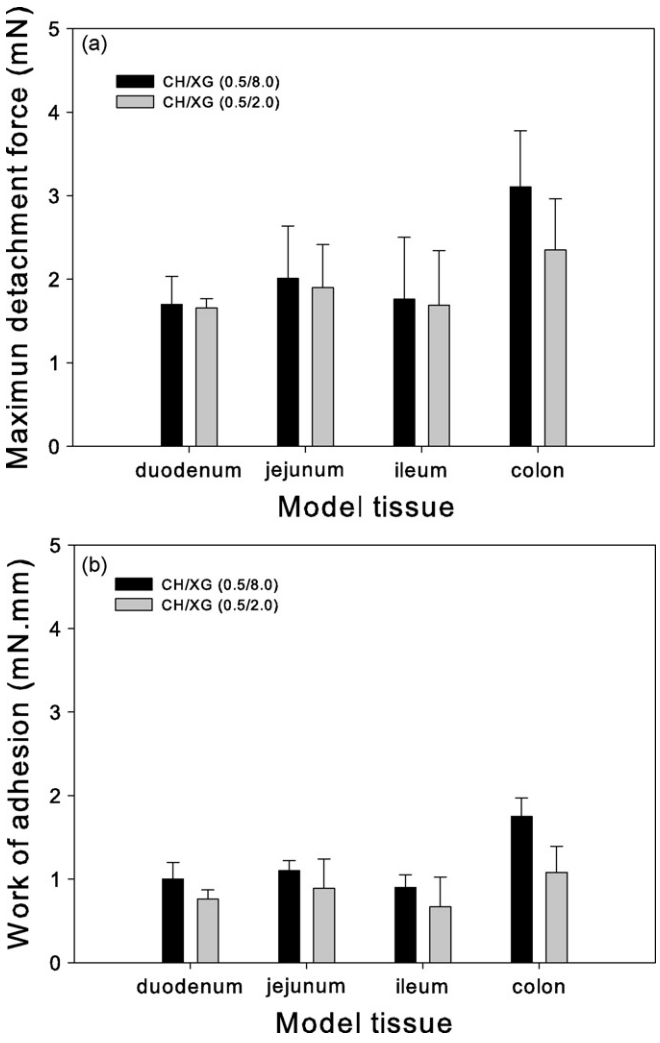

Fig. 6. *Ex vivo* mucoadhesive performance of phycocyanin tablets. Effect of GI mucosa on (a) maximum detachment force and (b) work of adhesion ($n=3$–5).

On the other hand, most of the studies have shown that the prerequisite for a good mucoadhesiveness of a polymer is the high flexibility of its backbone structure and polar functional groups. Such a flexibility of the polymer chains, however, is reduced if the polymer molecules are cross-linked each other. In accordance with this, the 0.5/8.0 SD chitosomes in colon mucose (Fig. 6) showed higher mucoadhesion than the chitosomes 0.5/2.0 FD that on the contrary showed a higher elasticity.

4. Conclusions

This work demonstrates the effects of formulation and process variables on particle size, drug content, swelling and drug release, and on the mucoadhesiveness of C-PC encapsulated liposomes coated by chitosan and xanthan gum hydrogel. The in vitro mucoadhesive study revealed that the SD method is advantageous to produce CP-P chitosomes (0.5/8.0 SD) with excellent mucoadhesive properties for colonic drug delivery. Moreover, these vesicles showed a regular shape and surface and the drug release is characteristic for a Fickian diffusion. Therefore, this formulation might be a good candidate for further research aimed to evaluate the effects

of C-PC on the inflammatory response in an experimental acute and chronic model acid-induced colitis in Wistar rats.

Acknowledgement

This study was supported by MIUR (Progetto DM28142) funds.

References

Barratt, G.M., 2003. Colloidal drug carriers: achievements and perspectives. Cell. Mol. Life Sci. 6, 21–37.
Benedetti, S., Benvenuti, F., Pagliarani, S., Francogli, S., Scoglio, S., Canestrari, F., 2004. Antioxidant properties of a novel phycocyanin extract from the blue-green alga Aphanizomenon flos-aquae. Life Sci. 75, 2353–2362.
Bhat, V.B., Madyastha, K.M., 2000. C-PC: a potent peroxyl radical scavenger in vivo and in vitro. Biochem. Biophys. Res. Commun. 275, 20–25.
Cevc, G., 2004. Lipid vesicles and other colloids as drug carriers on the skin. Adv. Drug Deliv. Rev. 56, 675–711.
Corrias, F., Dolz b, M., Herráez, M., Díez-Sales, O., 2008. Rheological properties of progesterone microemulsions: influence of xanthan and chitosan biopolymer concentration. J. Appl. Polym. Sci. 110, 1225–1235.
Diez-Sales, O., Dolz, M., Hernàndez, M.J., Casanovas, A., Herraez, M., 2007. Acyclovir delivery matrices based on poly(ethylene glycol)chitosan semi-interpenetrating networks. J. Appl. Polym. Sci. 105, 2121–2126.
Fiebrig, I., Harding, S.E., Rowe, A.J., Hyman, S.C., Davis, S.S., 1995. Transmission electron microscopy on pig gastric mucin and its interactions with chitosan. Carbohydr. Polym. 28, 239–244.
Glazer, A.N., 1994. Phycobiliproteins – a family of valuable, widely used fluorophores. J. Appl. Phycol. 6, 105–112.
Gonzalez, R., Rodriguez, S., Romay, C., Ancheta, O., Gonzales, A., Armesto, J., Remires, D., Merino, N., 1999. Anti-inflammatory activity of phycocyanin extract in acid acetic-induced colitis in rats. Pharmacol. Res. 39, 55–59.
Hejazi, R., Amiji, M., 2003. Chitosan-based gastrointestinal delivery systems. J. Control. Release 89, 151–165.
Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y., Oku, N., 2000. Application of surface-coated liposomes for oral delivery of peptide: effects of coating the liposome's surface on the GI transit of insulin. J. Pharm. Sci. 8, 248–252.
Leung, S.H.S., Robinson, J.R., 1990. Polymer structure features contributing to mucoadhesion II. J. Control. Release 12, 187–194.
Martínez-Ruvalcaba, A., Chornet, E., Rodrigue, D., 2007. Viscoelastic properties of dispersed chitosan/xanthan hydrogels. Carbohydr. Polym. 67, 586–595.
Mladenovska, K., Raicki, R.S., Janevik, E.I., Ristoski, T., Pavlova, M.J., Kavrakovski, Z., Dodov, M.G., Goracinova, K., 2007. Colon-specific delivery of 5-aminosalicylic acid from chitosan–Ca–alginate microcomplexes. Int. J. Pharm. 342, 124–136.
Nijenhuis, K., 1997. Thermoreversible network. In: Viscoelastic Properties and Structure Gels. Advances in Polymer Sciences. Springer, Berlin.
Nyström, B., Kjønijsen, A., Lindman, B., 1996. Effects of the temperature, surfactant, and salt on the rheological behavior in semidilute aqueous systems of a nonionic cellulose ether. Langmuir 12, 3233–3240.
Oechsner, M., Keipert, S., 1999. Polyacrylic acid/polyvinylpyrrolidone bipolymeric systems. I. Rheological and mucoadhesive properties of formulations potentially useful for the treatment of dry-eye-syndrome. Eur. J. Pharm. Biopharm. 47, 113–118.
Othes, S., Pire, R., 2001. Fatty acid composition of chlorella and Spirulina microalgae species. J. AOAC Int. 84, 1708–1714.
Pugh, N., Ross, S.A., Elsohly, H.N., Elsohly, M.A., Pasco, D.S., 2001. Isolation of three weight polysaccharide preparations with potent immunostimulatory activity from Spirulina platensis. Aphanizomenon flos-aquae and Chlorella pyrenoidosa. Planta Med. 67, 737–742.
Reddy, C.M., Bhat, V.B., Kiranmai, G., 2000. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from Spirulina platensis. Biochem. Biophys. Res. Commun. 277, 599–603.
Rimbbau, V., Caminis, A., Romay, C., Gonzales, R., Pallas, M., 1999. Protective effects of C-phycocyanin against kanic acid-induced neuronal damage in rat hippocampus. Neurosci. Lett. 276, 75–78.
Ritger, P.L., Peppas, N.A., 1987. A simple equation for description of solute release. I. Fickian and anomalous release from swellable devices. J. Control. Release 5, 23–36.
Romay, C., Armesto, J., Remirez, D., Gonzales, R., Ledon, N., Garcia, I., 1998. Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. Inflamm. Res. 47, 36–41.
Romay, C., Ledon, N., Gonzales, R., 1999. Phycocyanin extract reduces leukotriene B4 levels in arachidonic acid-induced mouse-ear inflammation test. J. Pharm. Pharmacol. 51, 641–642.
Romay, C., Gonzales, R., Ledon, N., Remirez, D., Rimbau, V., 2003. C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. Curr. Protein Pept. Sci. 4, 207–216.
Schumacher, U., Schumacher, D., 1999. Functional histology of epithelia relevant for drug delivery: respiratory tract, digestive tract, eye, skin and vagina. In: Mathiowitz, E., Chickering, D.E., Lehr, C.M. (Eds.), Bioadhesive Drug Delivery Systems: Fundamentals, Novel Approaches and Development, 1999. Marcel Dekker, New York, pp. 67–83.
Siepmann, N.A., Peppas, N.A., 2001. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Adv. Drug Deliv. Res. 48, 139–157.
Subhashini, J., Mahipal, S.V., Reddy, M.C., Reddy, M., Rachamallu, A., Reddanna, P., 2004. Molecular mechanisms in C-phycocyanin induced apoptosis in human chronic myeloid leukaemia cell line-K562. Biochem. Pharmacol. 68, 453–462.
Takeuchi, H., Yamamoto, H., Nuwa, T., Hino, T., Kawashima, Y., 1996. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. 13, 896–900.
Talukder, R., Fassihi, R., 2004. Gastroretentive delivery systems: hollow beads. Drug Dev. Ind. Pharm. 30, 405–412.
Thirawong, N., Thongborisute, J., Takeuchi, H., Sriamornsak, P., 2008. Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin–liposome nanomicrocomplexes. J. Control. Release 125, 236–245.
Tobyn, M.J., Johnson, J.R., Dettmar, P.W., 1995. Factor affecting in vitro gastric mucosa adhesion. I. Test conditions and instrumental parameters. Eur. J. Pharm. Biopharm. 41, 235–324.
Wittaya-areekul, S., Kruenate, J., Prahsarn, C.H., 2006. Preparation and in vitro evaluation of mucoadhesive properties of alginate/chitosan microcomplexes containing prednisolone. Int. J. Pharm. 312, 113–118.
Wong, C.F., Yuen, K.H., Peh, K.K., 1999. An in vitro method for buccal adhesion studies: importance of instrument variables. Int. J. Pharm. 180, 47–57.
Xu, Q., Tanaka, Y., Czernuszka, J.T., 2007a. Encapsulation and release of a hydrophobic drug from hydroxyapatite coated liposomes. Biomaterials 28, 2687–2694.
Xu, Y., Zhan, C., Fan, L., Wang, L., Zheng, H., 2007b. Preparation of dual crosslinked alginate–chitosan blend gel beads and in vitro controlled release in oral site-specific drug delivery system. Int. J. Pharm. 336, 329–337.
Yoshida, A., Takagaki, Y., Nishimune, T., 1996. Enzyme immunoassay for phycocyanin as the main component of Spirulina color in foods. Biosci. Biotechnol. Biochem. 60, 57–60.