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New environmental sensitive system for colon-specific delivery of peptidic drugs

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ABSTRACT

Nano and micro preparative technologies for the realization of pharmaceutical carriers represent an actual strategy for reaching the therapeutic success of drugs, particularly in the case of peptidic drugs. Vancomycin is here entrapped in carriers composed by a swellable, mucoadhesive and biodegradable albumin core, coated with fatty acids able to improve a colon-specific release. Bovine serum albumin nanospheres (core) were prepared from protein solutions using a coacervation method followed by thermal cross-linking at different temperature, or from protein solutions at different pHs using a coacervation method followed by thermal cross-linking at 75 °C. Solid nanospheres were collected by freeze-drying, loaded by soaking from solutions of vancomycin and subsequently coated with myristic, palmitic or stearic acid by spray-drying technique obtaining microcapsules. Nanosphere dimensions and polydispersity, drug loading capacity, swelling ability and mucoadhesion properties were evaluated, as well as in vitro release behaviour. The results indicated that nanospheres present an adequate loading capacity, a great swelling tendency and good mucoadhesion ability. Moreover, albumin cores showed a pH-dependent release according to the structure of thermally denaturated protein in different experimental conditions, while microcapsules showed a pH-dependent release according to the different fatty acids solubility in acidic and alkaline media.

1. Introduction

Colon specific drug delivery systems have gained increased importance for systemic delivery of drugs and also for the treatment of local diseases (Janovska et al., 2006; Van den Mooter, 2006). Well documented approaches to achieve colon-specific delivery included prodrugs (Jain et al., 2006), polymeric prodrugs (Sinha and Kumria, 2001), pH-dependent systems (Mahkam, 2007), time-dependent systems (Gazzaniga et al., 2006), and biodegradable systems (Musial and Kubis, 2005). Efficient colon drug delivery needs that the system only responds to the physiological conditions particular to the colon. Hence, new colon-specific delivery systems with improved site specificity and adequate drug release have been developed to accommodate different therapeutic needs. In particular, colon-specific delivery systems can be used to improve the bioavailability of protein and peptide drugs (Malik et al., 2007; Sinha et al., 2007). Vancomycin is indicated for the treatment of serious, life-threatening infections by Gram-positive bacteria which are unresponsive to other less toxic antibiotics and in the treatment of pseudomembranous colitis it must be given orally to reach the site of infection (Rizzello et al., 2003). To achieve successful colonic delivery, vancomycin needs to be protected from degradation in the environment of the upper gastrointestinal tract and then be abruptly released into the colon.

This work describes the use of thermally cross-linked bovine serum albumin (BSA) nanospheres coated with different fatty acids as carriers able to control the release of vancomycin in the colon environment responding strongly to stimuli such as pH, mucin interactions and enzyme digestion. Albumin cross-linking can be obtained either by self-cross-linking (direct reaction between functional groups in the polypeptide side chain) or by cross-linking agents such as glutaraldehyde and 2,3-butadione. Usually self-cross-linking is achieved by heating protein solution over 50 °C. Albumin nanospheres, having different physical state under known conditions, were prepared by coacervation method followed by heating. In particular BSA cores (nanospheres) were prepared from albumin solutions in distilled water (pH 6.5) using a coacervation method followed by thermal cross-linking at different temperature (60, 75 and 90 °C) or from BSA solutions at different pHs (2.0, 4.8 and 10.0; the ionic strength of buffers was adjusted to 0.2) using a coacervation method followed by thermal cross-linking at 75 °C. Solid nanospheres were collected by freeze-drying, loaded

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by soaking from solutions of vancomycin and subsequently coated with myristic, palmitic or stearic acids by spray-drying technique obtaining microcapsules. The structure of the different albumin cores was analyzed by Fourier transform infrared spectroscopy and correlated with nanosphere functional properties such as pH sensitivity in swelling, drug loading ability and drug release. Infact, different experimental conditions (charge distribution of albumin molecule in solution before coacervation and cross-linking temperature) can influence albumin cross-linking degree and consequently nanospheres behaviour in the different gastrointestinal conditions. Moreover, the pH sensitive coating can further modulate drug release in acidic and alkaline environments.

2. Materials and methods

2.1. Materials

Bovine serum albumin, vancomycin hydrochloride, myristic acid, palmitic acid and stearic acid were purchased from Fluka (Milan, Italy) and all the solvents employed were from Carlo Erba (Milan, Italy).

2.2. Determination of bovine serum albumin isoelectric point (pI)

2 g of albumin were dissolved in aqueous buffers (50 ml) having given pH values (2.0, 4.0, 4.8, 6.0, 8.0 and 10.0). The ionic strength of buffers was adjusted to 0.2. Albumin solutions were taken to measure absorbance at 600 nm using UV–vis spectroscopy (UV-1601, Shimadzu, Kyoto, Japan).

2.3. Preparation of BSA nanospheres denaturated at different temperatures

0.25 g of BSA were dissolved in 50 ml of distilled water (pH 6.5). Acetone was added dropwise to this solution at a rate of 1.0 ml/min (which was stirred continuously with a magnetic stirrer at room temperature), so as to produce a ratio 2:1 (v/v) acetone:BSA solution. Magnetic stirring (500 rpm) was continued at 60 °C (NS_A), 75 °C (NS_B) or 90 °C (NS_C) for 30 min to evaporate the acetone and yield an aqueous suspension of albumin nanospheres. Then nanosphere suspensions were cooled and processed by freeze-drying (Alpha 1-2, Christ, Naples, Italy).

2.4. Preparation of BSA nanospheres denaturated at different pHs

0.25 g of BSA were dissolved in 50 ml of aqueous buffers having the same ionic strength and different pHs: 2.0 (NS_D), 4.8 (NS_E) and 10.0 (NS_F). Acetone was added dropwise to this solution at a rate of 1.0 ml/min (which was stirred continuously with a magnetic stirrer at room temperature) so as to produce a ratio 2:1 (v/v) acetone:BSA solution. Magnetic stirring (500 rpm) was continued at 75 °C for 30 min to evaporate the acetone and yield an aqueous suspension of albumin nanospheres. Then nanosphere suspensions were cooled and processed by freeze-drying.

2.5. Dynamic light scattering

In order to evaluate nanosphere dimensions and stability in alkaline and acidic environments all the samples were suspended again in pH 1.2 HCl solution and pH 5.5 and 7.6 phosphate buffers and their size and polydispersity were monitored for 24 h by means of Photon Correlation Spectroscopy (PCS) (Berne and Pecora, 1976; Chu, 1974). Particle size and particle size distributions were measured using an instrument (Brookhaven 90-PLUS, New York, USA) with a He–Ne laser beam at a wavelength of 532 nm (scattering angle of 90°). Nanosphere suspensions were used for particle size measurement without filtering.

2.6. Fourier Transform Infrared Spectroscopy

The structure of albumin nanospheres was analyzed by Fourier Transform Infrared Spectroscopy. Spectra were recorded with a Jasco FT-IR-410 spectrophotometer (Lecco, Italy). Nanosphere samples were prepared by processing compressed KBr disks. To determine suitable compression conditions in order to avoid protein changes after compression with KBr, FT-IR spectra of native albumin were registered utilizing KBr disks compressed at different pressures and different protein dilutions (Chan et al., 1996.). Data obtained suggested that protein changes could be avoided by a suitable dilution of albumin in KBr (1 in 100) and a compression pressure of 1 tonne/cm^2.

2.7. Nanosphere swelling behaviour

In order to evaluated nanosphere swelling ability in different pH conditions particular to stomach, duodenum and colon disks approximately 20 mg in weight were prepared by a punch press working at 1 tonne/cm^2. The disks were immersed in 10 cm^3 volume pH 1.2 HCl solution or pH 5.5 and 7.6 aqueous buffers at 37 °C and weighed after 6 h (time corresponding at the higher swelling degree). The swelling ability (SW%) was determined according to the equation here reported:

$$SW\% = (Wh - Wd) \times \frac{100}{Wd}$$

where Wh is the weight of the hydrated disks and Wd is the initial weight of the dry disks.

2.8. Vancomycin loading and coating process

Vancomycin loading into nanospheres denaturated in different pH conditions (NS_D, NS_E and NS_F) was achieve by impregnation, soaking for 24 h at room temperature, under continuous stirring, 200 mg of nanospheres in a concentrate solution (100 mg of vancomycin in 100 ml of pH 10.0 phosphate buffer) acting as a swelling agent. The solvent was removed by ultracentrifugation (12,000 rpm, 30 min, 4 °C), samples were washed with the same buffer, immediately ultracentrifugated again and finally freeze-dried.

Loaded nanospheres were coated by spray-drying technique. Fatty acids (0.25 g of stearic, palmitic and myristic acids) and Span 60 (0.05 g) were dissolved in 50 ml of ethanol. These solutions were supplemented with 0.30 g of the different freeze-dried nanospheres (NS_D, NS_E and NS_F). The suspensions obtained were kept under agitation, nebulised and dried using a spray-drying apparatus (Büchi Mini Spray Dryed B-191, Flawil, Switzerland) in the following experimental conditions: inlet temperature 60 °C, outlet temperature 35 °C, air flow rate 600 NI/h and suspension flow rate 50 l/h.

All the coated nanospheres (microcapsules) produced by spray-drying ranged from 1 to 5 μm in diameter and showed regular shape and rough surface probably due to the presence of fatty acids in the coating material as observed using a scanning electron microscope (LEO 420, LEO Electron Microscopy Ltd., Cambridge, England).

2.9. Determination of drug content

An amount of 3.0 mg of uncoated nanospheres or coated nanospheres was weighed and transferred into a 3 ml volumetric flask and brought to volume with a 5% aqueous solution of

SDS (w/v). The suspension obtained was sonicated for 15 min, left at room temperature for 20 min and then centrifuged at 3400 × *g* for 15 min. The supernatant was finally diluted in water and analysed by capillary electrophoresis as described in a previous work (Musenga et al., 2006).

2.10. Mucoadhesion properties

The muco-adhesion behaviour was performed as described elsewhere (Cerchiara et al., 2003a,b). Briefly, 1 ml of a mucin suspension (0.05% w/v) was mixed with 1 ml of albumin nanospheres (0.1% w/v) for 24 h at pH 7.6 at 37 °C under continuous stirring. Mucin-nanoparticles interactions were evaluated by PCS.

In addition the residence time was determined. In particular, a freshly excised porcine intestinal mucosa (10 cm^2) was obtained from a local abattoir within 1 h of killing the animal and cleaned by washing with isotonic saline solution. 100 mg of loaded nanospheres were placed on mucosal surface, which was attached, with cyanoacrylate glue, over a polyethylene support fixed in an angle of 30° relative to the horizontal plane. The mucosa was thoroughly washed with phosphate buffer solution at the rate of 2 ml/min using a peristaltic pump. Sixty minutes after nanospheres placement on mucosal surface, the concentration of the drug in the collected perfusate was spectrophotometrically determined. The nanospheres amount corresponding to the drug amount in perfusate was calculated. The adhered nanospheres amount was estimated from the difference between the applied nanospheres amount and the flowed nanospheres amount. The ratio of the adhered nanospheres was computed as per cent mucoadhesion.

2.11. In vitro release studies

In vitro release profiles of vancomycin from the different uncoated and coated nanospheres were examined in pH 1.2 HCl solution or pH 5.5 and 7.6 phosphate buffers.

Alternatively in vitro release studies were conducted utilizing simulated gastric fluid (USP XXIV: pH 1.2, pepsin 0.32% w/v) and intestinal fluid (USP XXIV: pH 7.6, pancreatin 1.0% w/v) in order to evaluate the effect of proteolytic enzymes on the release behaviour.

The drug or the nanospheres were introduced into a donor cell containing 3 ml of the aqueous phase separated by a dialysis membrane (cellulose acetate, Mw cut off = 14.000, Delchimica Scientific Glassware) from a receiving compartment containing 10 ml of the same aqueous buffer, which was replaced after time intervals suitable to guarantee sink conditions throughout the runs. The studies were performed at 37 °C and the drug was detected in the receiving phase by HPLC utilizing a polar embedded C18 with TMS end-capping column (Sinergy 4u Fusion-RP 80A) and the following chromatographic conditions: mobile phase, phosphate buffer pH 7.0/ACN (90/10; v:v); flow rate, 1.0 ml/min; λ, 282 nm (Cerchiara et al., 2003a,b).

2.12. Statistical analysis

Variations in swelling, muco-adhesion and release data were tested using analysis of variance (ANOVA). In all cases, camparisons of the means of individual groups were performed using *t*-test. A significance level of $P < 0.05$ denoted significance in all cases.

3. Results and discussion

3.1. Determination of bovine serum albumin isoelectric point (pI)

The absorbance of albumin solution showed the highest value at pH 4.8 indicating that albumin molecules were in the aggregate state due to the low electrostatic repulsive force present at this pH. On the other hand surface net charge of albumin molecules increased as the pH value increased or decreased from 4.8 and high electrostatic repulsive force resulted in low absorbance value. From these results can be concluded that bovine serum albumin p*I* corresponds to 4.8 (Li et al., 2005).

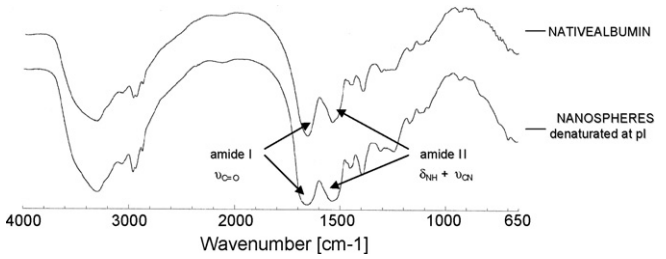

Fig. 1. FT-IR spectra of native albumin and nanospheres denaturated at the isoelectric point (NS_E).

3.2. Dynamic light scattering

All the freeze-dried nanospheres were readily redispersible in aqueous media. Good dimensional control in the range of 60–95 nm and very low polydispersity in the range between 0.01 and 0.05 were consistently obtained for all the nanospheres analysed. This finding indicated that lyophilisation did not cause aggregation of the nanospheres which were discrete entities. Moreover, nanosphere suspensions showed good stability in aqueous media during a period of 24 h. In particular, only a slight increase in particle size (approximately 5 nm) was observed probably due to nanosphere swelling tendency.

3.3. FT-IR analysis

Fig. 1 showed the IR spectra of native albumin and BSA nanospheres denaturated at the isoelectric point (NS_E). Table 1 reported the frequency position and peak intensity ratio of amide I and II bands (see Fig. 1) relative to all the nanospheres prepared. The peak intensity ratio is ratio of the band's intensity to CH_2 deformation vibration (1450 cm^{-1}) by the baseline method (Gorinstein et al., 1995).

As can be observed from Fig. 1 nanospheres denaturated at the isoelectric point presented the same characteristic peaks of native albumin relative to C=O stretching vibration (amide I band) and to a mixed vibration of N—H bending and C—N stretching (amide II band), but the band intensities and frequencies appeared modified. In particular, the peak intensity of both amide I and amide II bands decreased, the intensity ratio of the amide II band to the amide I band increases and the frequency position of both amide I band and amide II band shifted to the lower region.This spectral behaviour could be associated with the distruption of hydrogen bounds through heat denaturation and reflected a decrease in alfa-helix content of the denaturated protein.

Table 1
Band position of amide I and II and the intensity ratio (R_I, R_{II}) to the 1450 cm^{-1} band

	AMIDE I		AMIDE II	
	Band position (cm^{-1})	R_I	Band position (cm^{-1})	R_{II}
NS_A	1654.3	6.9	1544.0	4.7
NS_B	1653.4	6.8	1543.9	4.5
NS_C	1653.6	6.9	1543.2	4.5
NS_D	1653.2	6.2	1536.5	4
NS_E	1644.8	6	1526.9	3.5
NS_F	1650.2	7	1543.3	4.5

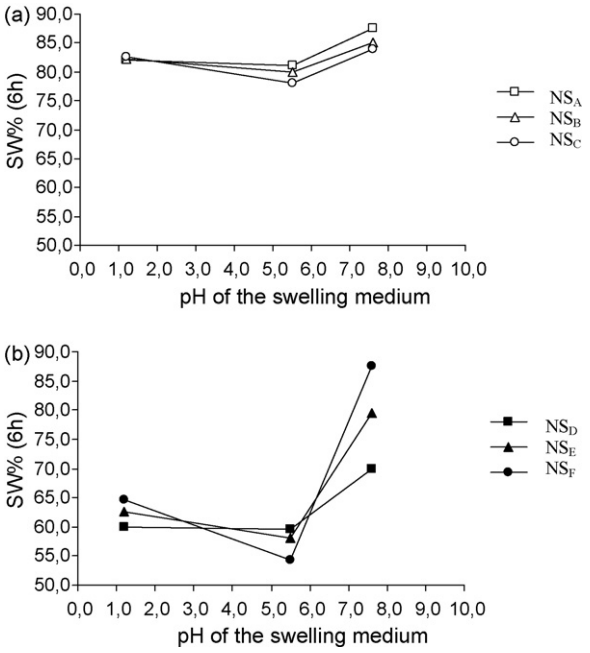

Fig. 2. Nanosphere swelling ability (SW%) in pH conditions particular to stomach (1.2), duodenum (5.5) and colon (7.6): (a) nanospheres denaturated at different temperature and (b) nanospheres denaturated in different pH conditions (the S.D. did not exceed the 5%).

Moreover, Table 1 shows that for the nanospheres denaturated at different temperature the peak position of both amide I and amide II bands and the peak intensity ratio of both amide I and amide II bands were similar, suggesting that an increase in denaturation temperature over 60 °C had no significant effect on cross-linking degree of the nanospheres (Shaheen et al., 2004). Instead for the nanospheres denaturated at different pHs the peak position of both amide I and amide II bands are at a minimum when the pH value is near the p*I* and the peak intensity ratio of both amide I and amide II bands increased when the pH value differed from p*I*. From these results it could be concluded that the structure of nanospheres denatured at the isoelectric point presented the lowest number of hydrogen bounds and the lowest alfa-helix content, that is to say the highest cross-linking degree.

3.4. Nanosphere swelling behaviour

Fig. 2 reported nanosphere swelling ability in pH conditions particular to stomach (1.2), duodenum (5.5) and colon (7.6): (a) nanospheres denaturated at different temperature and (b) nanospheres denaturated in different pH conditions. Fig. 2(a) showed that nanospheres denaturated at different temperature have similar swelling ability in different pH condition confirming their analogous cross-linking degree. Instead, Fig. 2(b) revealed that nanospheres denaturated in different pH conditions showed a minimum water uptake at pH near the isoelectric point. This behaviuor can be attributed to the decreased amount of net charge which affected nanosphere swelling ability.

Moreover, NS_D, NS_E and NS_F presented a pH sensitive swelling. In particular,nanospheres denaturated at alkaline pH showed the highest sensitivity among the samples as denaturation at this particular pH requires less amounts of carboxyl groups for the cross-linking. Infact, the contribution of amino groups to the pH sensitive mechanism can be ignored because there are much more carboxylic groups than amino groups in native albumin molecules and also in denaturated nanospheres.

Table 2
Drug Loading (DL w/w, %) and Entrapment Efficiency (EE%) for the uncoated nanospheres (NS_D, NS_E, NS_F) and for the relative microcapsules ($n = 3$, the S.D. did not exceed the 5%)

	DL w/w, %			EE %		
	NS_D	NS_E	NS_F	NS_D	NS_E	NS_F
Uncoated NS	26.8	23.5	30.3	53.6	47.0	60.6
Microcapsules (myristic acid)	12.9	10.9	13.6	96.2	92.8	89.8
Microcapsules (palmitic acid)	11.5	11.0	14.5	85.8	93.6	95.7
Microcapsules (stearic acid)	12.1	11.4	14.9	90.3	97.0	98.3

3.5. Determination of drug content

Drug loading (DL w/w, %) was calculated as the ratio mass of vancomycin in particles/mass of particles recovered × 100. Entrapment efficiency (EE%) was used to estimate the technological process suitability and was calculated both for nanospheres preparation and for microcapsules preparation (coating process) as follows:

EE% (nanospheres) = (mass of vancomycin in nanospheres/mass of vancomycin used for the preparation of nanospheres) × 100
EE% (microcapsules) = (mass of vancomycin in microcapsules/mass of vancomycin present in uncoated nanospheres) × 100

The mean values for three replicate determinations are reported in Table 2.

Data suggested that vancomycin can interact with hydrated albumin cores during the soaking process. In particular nanospheres denaturated at alkaline pH (SN_F) provided the highest drug loading due to their greatest swelling tendency among the samples. Moreover, the coating process produced microcapsules with good entrapment efficiency (in the range of 85.8–98.3%) indicating that spray-drying is a suitable technique for albumin nanosphere encapsulation.

3.6. Mucoadhesion properties

From the data reported in Fig. 3 can be observed that mucin/nanosphere aggregates presented higher dimensions than the standard samples. This behaviour suggests that albumin nanospheres can interact with mucin molecules by Van der Waals forces, electrostatic interactions, or hydrogen bounds.

In addition, the nanospheres adequately adhere on intestinal mucosa. Infact, 60 min after nanospheres placement on mucosal surface, the percentages of mucoadhesion were 85% for NS_D, 76% for NS_E and 88% for NS_F. These results may be correlated to the amounts of free functional groups involved in the swelling mechanism and

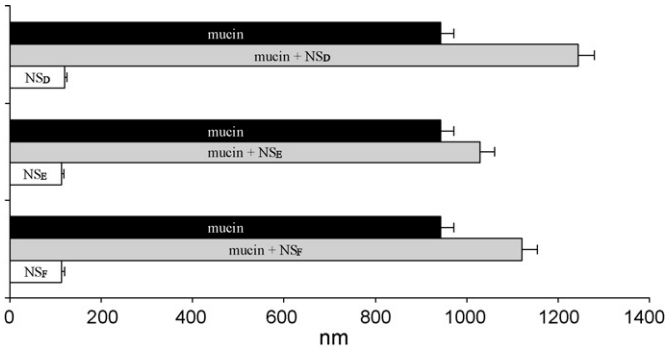

Fig. 3. Mean size of nanospheres (NS_D, NS_E, NS_F) with or without mucin in pH 7.6 acqueuos buffer at 37 °C ($n = 5$, mean ± S.D.).

also in the mucoadhesive interactions. Infact, nanospheres denaturated at alkaline pH which present a less cross-linked structure (major amount of free functional groups) provided the highest swelling and mucoadhesive ability.

3.7. In vitro release studies

In vitro release studies from uncoated nanospheres were performed at different pHs (Fig. 4). The attention was focused on the nanospheres denaturated at alkaline pH which presented the highest drug loading ability and the greatest pH sensitivity to swelling. From the release profiles at pH 1.2 can be observed that nanospheres denaturated at alkaline pH provided higher drug release than other samples. This behaviour is not acceptable for a colon-specific system which should be able to limit drug release in the stomach environment, but can be avoided by coating albumin cores with fatty acids. At pH characteristic of duodenum (5.5) nanospheres denaturated at alkaline pH showed lower drug release than other samples. This behaviour fits with the therapeutic needs of vancomycin whose release should be limited in the upper part of intestine. At pH characteristic of the colon (7.6), nanospheres

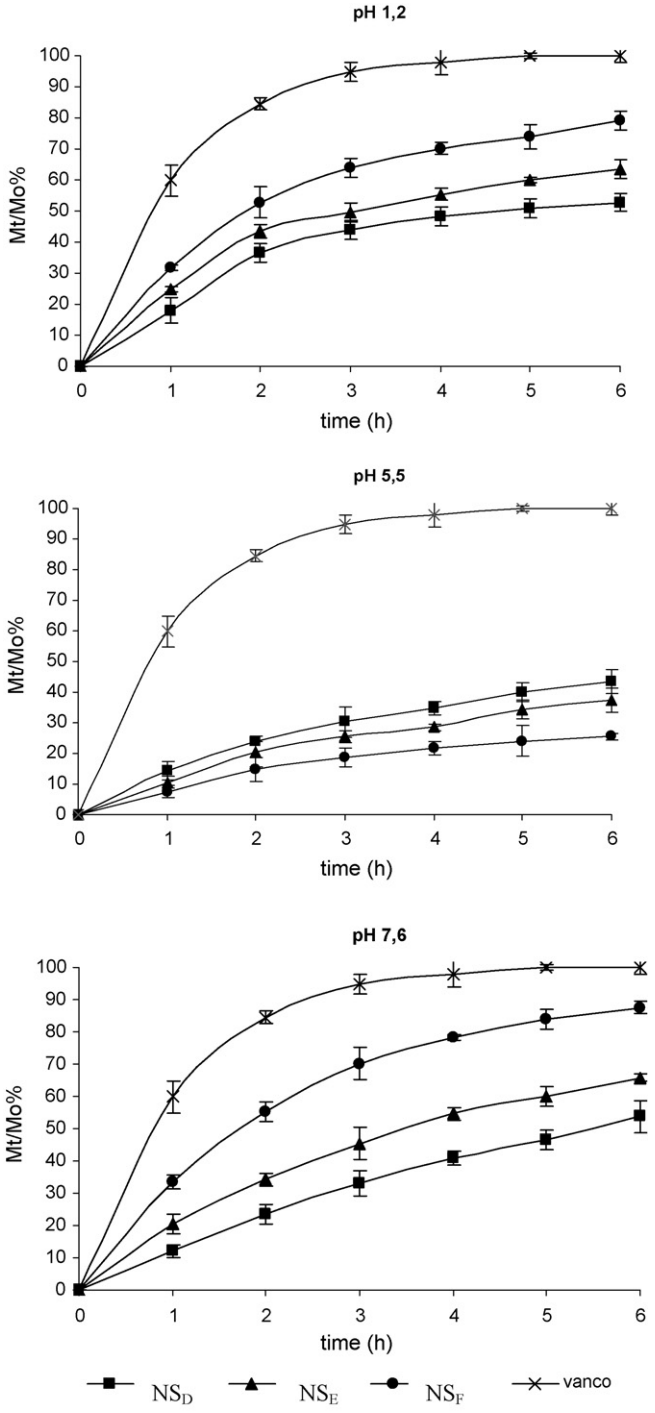

Fig. 4. Vancomycin dissolution profiles (vanco) and vancomycin release from uncoated nanospheres (NS_D, NS_E, NS_F) in different pH conditions (pH 1.2, 5.5 and 7.6). Mt/Mo corresponds to vancomycin fractional amount released at each time ($n = 3$, mean ± S.D.).

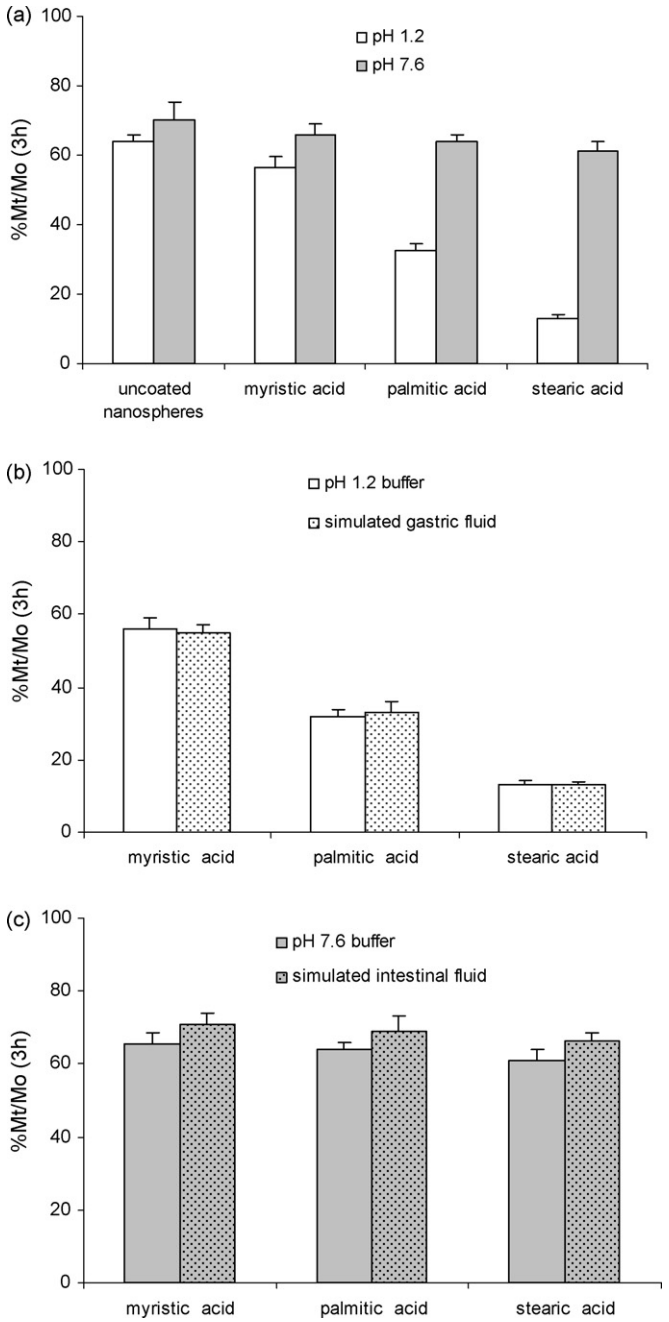

Fig. 5. Vancomycin release from coated nanospheres (NS_E) in different pH conditions (pH 1.2 and 7.6 (a) and in simulated gastric (b) and intestinal (c) fluids. Mt/Mo(3h) corresponds to vancomycin fractional amount released after 3 h ($n = 3$, mean ± S.D.).

denaturated at alkaline pH provided higher drug release than other samples. This is an adequate behaviour for vancomycin whose availability should be improved in the colonic environment. Finally, these data showed that exist a good correlation between fractional amount released and nanosphere swelling ability.

In vitro release studies from coated nanospheres were performed at pH 1.2 and pH 7.6. Fig. 5(a) showed that drug availability at pH 1.2 was drastically reduced by the coating, particularly in the presence of stearic acid (which is the longest chain fatty acid). Instead, vancomycin release at pH 7.6 was not hindered by the coating.

In vitro release studies were also performed utilizing simulated gastric fluid (USP XXIV: pH 1.2, pepsin 0.32% w/v) and simulated intestinal fluid (USP XXIV: pH 7.6, pancreatin 1.0% w/v). Fig. 5(b) showed that the presence of pepsin in gastric fluids did not modify vancomycin release showing the coating ability to protect albumin core. Instead the results reported in Fig. 5(c) indicate that the presence of pancreatin in intestinal fluids improved drug release due to albumin core destabilization.

4. Conclusions

The good mucoadhesion properties of uncoated albumin nanospheres could improve vancomycin availability prolonging the permanence of the drug on the intestinal mucosa. Moreover, nanospheres obtained by albumin cross-linking at alkaline pH and coated with fatty acids, particularly stearic acid, showed low drug release in the stomach environment and adequate release in intestinal fluids and can be considered good candidates for colon-specific delivery of peptidic drugs.

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