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In vitro evaluation and modification of pectinate gel beads containing trimethyl chitosan, as a multi-particulate system for delivery of water-soluble macromolecules to colon

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Abstract

Pectinate beads containing trimethyl chitosan chloride (TMC) as an absorption enhancer were prepared using Coomassie Brilliant Blue G 250 (CB) as a relatively high molecular weight water-soluble model drug. Effects of different formulation variables, such as cross-linker type, cross-linking time, cross-linker concentration, TMC: pectin ratio, pectin concentration and voltage of the bead generator, were assessed on in vitro bead characteristics by release and swelling–erosion studies. The bead formulation was optimized by factorial design. Some measures were taken to improve the bead characteristics and prolong their integrity during the gastrointestinal transit, such as biomineralization of the beads or coating them with high-methoxy pectin (PHM) or Eudragit L30-D 55 (EU). Possible CB-TMC complexation was investigated by Job's Plot method. The suitable system was obtained by coating the optimized core formulation with PHM or EU. TMC was found to form a complex with CB as a model anionic drug. Therefore, TMC-drug interactions can be used to modify the release characteristics of dosage forms.

Keywords: Pectinate bead; Swelling–erosion study; Trimethyl chitosan; Colon-specific delivery; Water-soluble drugs

1. Introduction

In recent years colon-specific drug delivery has received much attention. This is in part due to the spiraling trend of introduction of new peptide and protein drugs to the market and the need for their oral delivery (Zhang, Alsarra, & Neau, 2002). However, so far it has not been possible to administer these new and effective entities via the oral route due to their sensitivity to the acidic environment of the stomach and degradation by the proteolytic enzymes of the upper GI tract. Above all, owing to their high molecular weights and hydrophilic nature, peptides and protein drugs are scarcely absorbed.

Colon is a relatively mild environment with reduced proteolytic activity and decreased fluid and motility compared with the small intestine, which could afford advantages in terms of incorporating multiple components in the formulation, such as absorption enhancers that must reach the epithelial absorptive layer in a high concentration and in close spatial proximity (Mathiowitz, 1999); therefore, there will be hopes for successful oral administration of peptide and protein drugs, provided that they would reach the colon unharmed, and their absorption would be enhanced in colon.

Moreover, the concepts of delayed- or sustained-release dosage forms and local drug therapy have also contributed to the extensive research on the colonic route of drug administration. Several approaches are proposed for successful delivery of drugs to colon, such as pH-dependant and time-controlled systems, pressure-controlled devices and microbially triggered ones (Yang, Chu, & Fix, 2002).

Due to the large population of microflora in colon, the microbially triggered systems seem more specific for colonic drug delivery (Vandamme, Lenourry, Charrueau, & Chaumeil, 2002). The colonic microflora can degrade the polysaccharides, such as pectin.

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Pectin is one of the most widely investigated polysaccharides in the colon-specific drug delivery. It is predominantly a linear polymer of mainly α-(1–4)-linked D-polygalacturonic acid residues. It has been used in different dosage forms for colon-specific drug delivery. Pectin can be broken down by pectinase enzymes produced by anaerobic bacteria of colon and control the drug release by this principle. It can also act via the pH- and time-controlled mechanisms. However, due to water solubility and swelling, pectin is not capable of shielding the drug effectively during the passage through the stomach and small intestine. Therefore, less water-soluble forms of pectin should be used in this regard (Sinha & Kumria, 2001). For instance, low-methoxy pectin (degree of methoxylation $<50\%$) can form gel beads with an 'egg-box' configuration, with reduced solubility and swelling, in the presence of many divalent cations (Wellner, Kačuráková, Malovíková, Wilson, & Belton, 1998). Moreover, introduction of amide groups into low-methoxy pectin molecule tends to make the pectin less hydrophilic, increasing the tendency to form gels (CP Kelco information sheet; Wakerly, Fell, Attwood, & Parkins, 1997). As a result, by utilizing amidated low-methoxy pectin, more resistant beads can be prepared.

Beads have several advantages over single-unit dosage forms: they are multiple-unit dosage forms with established merits such as predictable and reproducible gastrointestinal transit time (especially for gastric residence time which is the major variable in overall gastrointestinal transit time), more consistent blood levels and improved bioavailability of the embedded drug, less localized gastrointestinal disturbances and greater product safety (Mathiowitz, 1999; Pillay & Fassihi, 1999a). They are prepared easily and quickly in ranges of 50–2000 μm with perfect spherical shape and narrow size distribution, without any use of organic solvents and harsh ingredients and time-consuming procedures.

Some researchers have studied different aspects of pectinate beads; Munjeri, Collett, and Fell (1997) investigated the release of indomethacin and sulphamethoxazole from calcium pectinate beads (uncoated or coated with chitosan) in different pH values. Sriamornsak and Nunthanid (1998) and Sriamornsak (1998, 1999) studied the characteristics of pectinate beads containing indomethacin as a water-insoluble model drug or albumin. Pillay et al. (1999a,b) examined the behavior of calcium alginate, calcium pectinate and calcium–alginate–pectinate in various buffers using diclofenac sodium as a model drug. El-Gibaly (2002) investigated delayed-release zinc pectinate ketoprofen-loaded beads formed into single-unit tablets. However, few studies have addressed the inclusion of relatively high molecular weight and water-soluble drugs (such as the situation encountered with peptide drugs) in the beads. Moreover, with the exception of the study by Pillay and Fassihi (1999a), in the previous studies continuous pH changes, simulating gastrointestinal pH variation, have not been used. Besides, in these studies the capability of the beads to reach colon intact and as a multiple-unit dosage form is not investigated. Sometimes the release data alone can be misleading with regards to preservation of the physical shape of a dosage form; for instance the beads may lose their spherical structure and consistency and become a shapeless single-unit gel in the simulated gastrointestinal media, but at the same time they may have kept the major amount of the drug unreleased. Therefore, apart from the release studies, evaluation of swelling behavior of beads is essential to determine their in vivo fate and integrity of shape and structure throughout the gastrointestinal tract and upon reaching colon. On the other hand the swelling studies elucidate the mechanisms of drug release. During the swelling studies, the weight changes do not always follow an increasing trend and upon release of drug and erosion of the polymeric matrix, negative changes are also observed, hence the term swelling–erosion ratio (SER) was coined and employed throughout this study. This work was designed to assess the feasibility of pectinate beads to protect and deliver a water-soluble high molecular weight model drug to colon in a multi-particulate form.

Oral drug delivery of high-molecular weight hydrophilic agents needs to be coupled with the use of an absorption-enhancing agent, which is safe and biodegradable. Chitosan is an established mucoadhesive and absorption-enhancing polymers, but it has a pk_a of 5.5, thus it is only soluble in acidic environments, where most of the amino groups are protonated. Recent studies have shown that only protonated soluble chitosan opens the tight junctions, facilitating the paracellular transport of hydrophilic compounds. To overcome this problem a chitosan derivative, *N*, *N*, *N*-trimethyl chitosan chloride (TMC), has been synthesized and characterized. TMC shows higher solubility than chitosan in a broader pH range and is proved to be non-toxic (Thanou, Kotzé, Verhoef, Brussee, & Junginger, 2000). Therefore, in this study, TMC was included in the beads and its reciprocal effect on the pectinate beads characteristics was investigated.

This work focuses on in vitro behavior of pectinate beads with different formulation variables and modification of these beads to optimize their characteristics for colon-specific drug delivery of water-soluble drugs.

2. Experimental

2.1. Materials

Low-methoxy amidated pectin type GENU® pectin LM-104 AS-FS (PLMA) (D.M. = 28%, D.A. = 20%) was purchased from CPKelco (Denmark). Chitosan type Chitoclear FG95 (D.D. > 95%) was supplied by Primex (Norway). Pectinex Ultra SPL (pectinase from *Aspergillus aculeatus*, activity 26000 PG/ml at pH 3.5) and high-methoxy pectin from citrus fruits (PHM) were purchased

from Sigma, USA. Calcium chloride (Ca chloride), zinc acetate (Zn acetate), *n*-methyl-2-pyrrolidone (NMP), methyl iodide, sodium iodide (NaI), Coomassie Brilliant Blue G 250 (CB), di-sodium hydrogen phosphate (Na_2HPO_4), potassium hydrogen phthalate, polyethylene glycol 600 (PEG600) and triethyl citrate (TEC) were obtained from Merck, Germany. Eudragit L 30-D 55 (EU) was a gift from Röhm Pharma Polymers, Germany and Akbarieh Co., Iran. All other chemicals used were of reagent grade.

2.2. Synthesis of TMC

TMC was synthesized according to the method by Sieval et al. with slight modifications (Sieval, Thanou, Kotzé, Brussee, & Junginger, 1998). Briefly, 2 g of chitosan was dissolved in 80 ml of NMP and stirred in a 2-necked flask in a constant temperature water bath at 60 °C. The flask was connected to a condensation column. 11 ml of 15% NaOH solution was added to the flask and it was followed by addition of 11.5 ml of methyl iodide (as the methylation agent) and 4.8 g of NaI. The mixture was stirred for 75 min and precipitated with addition of 200 ml of ethanol, centrifuged, washed with acetone on a sintered glass filter and dried. In the second stage, 80 ml of NMP was added to the precipitate (mainly dimethyl chitosan iodide) and the mixture was stirred at 60 °C. Then 11 ml of 15% NaOH, 7 ml of methyl iodide and 4.8 g NaI were added successively and the mixture was stirred for 30 min. An additional 2 ml of methyl iodide and 0.6 g pellets of NaOH were added and stirring was continued for 1 h. This mixture was precipitated with 200 ml of ethanol, centrifuged and the solid substance was filtered on a sintered glass filter and washed with acetone to obtain a powdery substance, which is trimethyl chitosan iodide. To exchange iodide with chloride, trimethyl chitosan iodide was dissolved in 40 ml solution of 10% NaCl. The solution was precipitated with 200 ml of ethanol. Excess ethanol should be avoided because it will also precipitate NaCl. The mixture was centrifuged and the supernatant containing the excess NaCl was completely removed. The precipitate, which was TMC, was filtered and washed with acetone, dried and milled to obtain an off-white water-soluble powder. To determine the degree of quaternization (% DQ), ^{1}H NMR spectrum of TMC was measured in D_2O, using a 400 MHz spectrometer (Varian 400 Unityplus spectrometer, USA). The %DQ was calculated from Eq. (1):

$$\%\mathrm{DQ} = (\textstyle\int \mathrm{TMC}/\int H) \times 1/9 \quad (1)$$

Where $\int$TMC is the integral of the trimethyl amino group (quaternary amino) peak at 3.3 ppm on the ^{1}H NMR spectrum, and $\int$H is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm on (Snyman, Hamman, Kotzé, & Rollings, 2002).

2.3. Preparation of pectinate gel beads

PLMA and CB, with or without TMC, were dissolved in distilled water at the desired concentrations indicated in each experiment. The gel mixture was completely dearated under vacuum (Fast Vac™ vacuum pump, J/B Industries, USA) and employed to prepare the beads by ionotropic gelation using an electrostatic bead generator (Nisco encapsulator VAR V-1, Switzerland) equipped with a syringe pump (Kd Scientific, USA). The mixture was dropped from a syringe into a cross-linking solution (either Zn acetate or Ca chloride), at the rate of 10 ml/h. The nozzle diameter was either 0.7 or 1.1 mm. The dropping distance was 5 cm. Volume of the cross-linking solution was 25 ml for every 10 ml of the gel mixture. The prepared beads were cross-linked for the period stated in each experiment, then washed twice with distilled water and dried overnight at room temperature.

2.3.1. Rationale for selection of ingredients and processes; preliminary studies

Some preliminary studies were first carried out to determine the appropriate formulation variables for the preparation of beads.

PLMA was chosen for this study because of its capability to form beads (compared to PHM). Moreover the introduction of amide group into its structure makes it less hydrophilic-increasing the tendency to form gels (compared to conventional low-methoxy pectin) (CP Kelco information sheet; Wakerly, Fell, Attwood, & Parkins, 1997). PLMA concentrations of 3.5, 5 or 7% (w/v) were initially studied for the preparation of beads. CB (M = 854.03 g/mol) was chosen as a model drug for relatively high molecular weight and water-soluble therapeutic agents. CB concentration of 0.3% (w/v) in the PLMA gel was selected, based on the experiments of limit of quantification of CB with spectrophotometric method, and the volumes of the cross-linking solution and release media, for easy assay of the drug.

Zn acetate or Ca chloride at the concentrations of 0.15 or 0.6 M were chosen as cross-linker for the beads and were compared to each other. To assess the safety of application of Zn acetate, some measures were taken. In order to assess the amount of Zn^{2+} retained in the beads, precise 10-ml volumes of PLMA gel mixture of 3.5 or 5% were used to prepare the beads with Zn acetate 0.15 M as the cross-linker. The obtained beads were washed with distilled water and completely dried until no further weight change was observed. The beads were then weighed with an analytical balance with readability of 0.0001 g (OHAUS GA200, Germany), and their weight was compared with the dry content of pectin in the initial gel volume.

Cross-linking times (t_c) of 2, 4 or 24 h were investigated. In addition different TMC:PLMA ratios (1:8, 1:6 and 1:4) were investigated and the suitable ratio was selected based on the preliminary studies.

The beads were prepared with an electrostatic bead generator. Voltage of the bead generator can be changed based on the desired bead characteristics. Since the electrode, which is placed in the cross-linking solution, has a positive charge, it might repel Zn^{2+} or Ca^{2+} and decrease the local concentration of the cross-linker and consequently diminish the cross-linking of the beads. Voltages of 0 or 2 kV were chosen to prepare the beads of the same size and compared them to determine the effect of the encapsulator voltage on the beads.

2.3.2. Experimental design and optimization of beads

Based on the results of the preliminary experiments (see Section 3), a 2^3 full factorial design was used to prepare the optimized beads and assess the effect of PLMA concentration, Zn acetate concentration and voltage of the bead generator on bead characteristics (Table 1).

2.4. Determination of loading efficiency

Loading efficiency (LE) of the beads for CB was determined by the indirect method, i.e. exact volumes of PLMA gel mixture containing CB were employed to prepare beads and the amount of CB lost in 25 ml of cross-linking solution was assayed spectrophotometrically at 578.6 nm (Scinco, S-3100 spectrophotometer, Korea). LE of the beads for CB was calculated by Eq. (2):

$$LE_{CB} = [(M_T - M_L)/M_T] \times 100 \quad (2)$$

Where M_T is the theoretical amount of CB in the beads and M_L is the amount of CB lost in the cross-linking and washing solutions.

LE of the beads for TMC was determined by a simple gravimetric method, using Eq. (3):

$$LE_{TMC} = (W_{TMCO}/W_{TMCT}) \times 100 \quad (3)$$

Where W_{TMCT} is theoretical dry weight of TMC used in the beads preparation and W_{TMCO} is the weight of the loaded TMC. W_{TMCO} in turn is calculated from Eq. (4):

$$W_{TMCO} = W_O - W_{(P+CB)} \quad (4)$$

W_O is the observed weight of beads and $W_{(P+CB)}$ is the sum of dry weight of pectin and dry weight of loaded CB (calculated by the above-mentioned method).

2.5. Drug release behavior of beads in simulated gastrointestinal media

In standard methods of dissolution experiments in pharmacopoeias, due to the large volumes of the vessels, relatively large amounts of multi-particulate dosage forms are needed; whereas in our study, the beads could not be prepared in large amounts due to the limitations of the laboratory-scale bead generator and shortage of the ingredients, particularly trimethyl chitosan, which was synthesized by us. On the other hand if small amounts of beads in a standard dissolution tester were to be used, very high amounts of the model drug, Coomassie Brilliant Blue G 250 in the beads, should have been incorporated to the beads, to enable the assay the amount of drug released, in the high volumes of the buffers. This was not either feasible, because it adversely affected the beads' characteristics. Therefore, in this study, a modified alternative method, used by some other research groups for their dissolution experiments of multi-particulate dosage forms, intended for oral delivery (Zhang, Alsarra, & Neau, 2002), was employed.

In one set of experiments, fixed amounts of beads were placed in test tubes containing 10 ml of USPXXIII acidic buffer medium (KCl/HCl, pH 1.5) in an oscillating water-bath (Memmert, Germany) at 37 °C (for 2 h). Then the medium was replaced with 10 ml of USPXXIII phosphate buffer solution (KH_2PO_4/NaOH, pH 7.4) and was further oscillated in the water-bath for 4 h. In another set of experiments, the beads were placed directly in phosphate buffer medium (pH 7.4) for 4 or 5 h to assess the beads' characteristics in simulated intestinal environment alone. In the third set of experiments the beads were first brought in

Table 1
2^3 factorial design utilized for preparation of pectinate beads

Formulation code[a]	Independent variables			Dependent variables	
	PLMA concentration (% w/v)	Zn acetate concentration (M)	Voltage (kV)	Rel_{4h}(%)[b]	SER_{4h}(%)[c]
F1	3.5	0.15	0	19.2±5.3	176.6±70
F2	5	0.15	0	6.17±2.8	150±43.5
F3	3.5	0.6	0	26.1±4.3	−86±15.2
F4	5	0.6	0	4.8±1.2	26.6±15.2
F5	3.5	0.15	2	15±2	73.3±46.1
F6	5	0.15	2	2.9±1.3	146.6±75.7
F7	3.5	0.6	2	28±6.5	−86.6±23
F8	5	0.6	2	3.1±0.4	−63.3±15.2

[a] t_c = 2 h, TMC:PLMA ratio = 1:6, nozzle diameter = 0.7 mm.
[b] Cumulative percent CB released in 4 h from beads in phosphate buffer pH 7.4. Mean ± S.D., n = 3.
[c] Swelling–erosion ratio (SER) of beads in 4th h in phosphate buffer pH 7.4. Mean ± S.D., n = 3.

contact with 10 ml of acidic buffer solution (pH 1.5) for 2 h, followed by 4 h in 10 ml of phosphate buffer medium (pH 7.4). Afterwards, the beads were transferred to USPXXIII phthalate buffer media (pH 5) with or without 50 μl of Pectinex Ultra SPL and the experiments were continued for another 3 h. A pH of 5 was chosen for colonic medium, as a compromise between colonic pH (6.4–7.0) and the optimum pH for enzyme activity (3–5) (Macleod, Fell, & Collett, 1999). With regards to the low pH value chosen for colon, pH values, as low as 5.5–5.7 are also reported for colon; therefore a pH value of 5 is not very far from the actual colonic pH (Semdé, Moës, Devleeschouwerm, & Amighi, 2003; Fallingborg, 1999). Some researchers have used this pH to assess the effect of pectinolytic enzymes on pectin-containing dosage forms, intended for colon-specific drug delivery (Macleod, Fell, & Collett, 1999; Semdé et al., 2003), but one should note that these conditions may somewhat weaken the relevance to colonic environment. The periods of time, chosen for exposure of beads to each media, are almost the gastrointestinal condition encountered in vivo (Goto et al., 2004; Raghavan, Muthulingam, Jenita, & Ravi, 2002).

One test tube was assigned to each time point. The samples of 5 ml were withdrawn at desired time points, centrifuged at 4000 rpm for 10 min, and assayed for CB against the respective blank buffers. Sink condition was maintained through out the experiments (based on solubility of CB in the respective media). The samples were assayed spectrophotometrically at 578.6 nm and CB concentration was determined using calibration curves in the respective media.

2.6. Swelling–erosion behavior of the beads

In these experiments, pre-weighed amounts of dry beads were placed in the same conditions stated for the release studies. One test tube was assigned to each time point. The beads were removed at every time point, blotted on filter paper to remove the excess water and weighed. The swelling–erosion ratio (SER) was calculated by Eq. (5):

$$\mathrm{SER} = [(W_t - W_0)/W_0] \times 100 \quad (5)$$

W_t is the beads' weight at the given time point, W_0 is the initial weight of the dry beads.

Positive SER and upward trend denote overall swelling and weight gain of beads with water absorption, while negative SER and downward trend demonstrate the erosion and/or drug release of beads. SER −100% is when the beads either are in a form of a shapeless loose gel, which cannot be manipulated for weighing, or have dissolved completely.

2.7. Modification of pectinate beads

To improve the stability of beads in phosphate buffer after exposure to acidic medium, some measures were adopted as follows. Release studies and swelling–erosion experiments were performed on these modified beads. These beads were prepared based on the optimized core formulation obtained from the results of the factorial design.

2.7.1. Biomineralization of beads

Biomineral-inspired beads have been prepared with alginate/chitosan with enhanced mechanical strength and reduced permeability in deionized water (Leveque, Rhodes, & Mann, 2002). The concept of biominerlization was exploited to prepare biomineralized Zn phosphate pectinate beads.

In situ precipitation of phosphate salts around pectinate beads was carried out. Na_2HPO_4 at 50 or 100 mM was added to pectin gels and the beads were prepared as described in Section 2.3. Upon preparation of beads, in situ spontaneous precipitation of $ZnHPO_4$ membranes occurred around the beads.

2.7.2. Coating the beads with PHM

PHM is known to swell less than PLMA, because the ester ($COO(CH_3)$) groups present in PHM are less hydrophilic than the acid (COO^-) groups of PLMA (CPKelco information sheet; Vandamme et al, 2002). Therefore, in order to protect the core beads from exposure to acidic environment and control their swelling, the beads were coated with 3 or 5% (w/v) solutions of PHM. Briefly the dry beads were added to solutions of PHM upon stirring and after 15 min they were removed, washed gently with distilled water and dried at room temperature overnight.

2.7.3. Coating the beads with EU

The beads were coated with EU, a pH-dependant enteric polymer (which dissolves at pH values over 6) by a suspension-salting out method (SSO) designed in our laboratory. EU is known to coagulate in presence of Mg stearate (Röhm Pharma Polymer's Literature). We exploited this knowledge and used NaCl (a neutral salt) to design a method for coating beads with EU.

Briefly the dried beads were pre-treated with 25% (w/v) solutions of NaCl (an agent that induces the salting out of EU). The slightly damp beads were suspended in a solution of PVA 1% (w/v) (a protective colloid which prevents the beads from adhering together) (Lin, Chen, & Teng, 1999) and stirred under the propeller mixer (Velp Scientifica, Italy) for 1 min. Then 5 ml dispersion of EU containing 5% (w/v) PEG 600 and 5% (w/v) TEC (as plasticizers), was added and the suspension was stirred for 5 min. The coated beads were washed with distilled water and dried.

2.8. Assessment of CB-TMC complexation

During the studies it was noted that inclusion of TMC in the formulations increases the loading efficiency and modifies the release of CB, while TMC is a water-soluble ingredient and believed at first to increase the release and disintegration of the beads. Therefore, the possibility of

formation of a complex between TMC and CB was considered. In order to examine TMC-CB interaction, a continuous variation technique (Job's plot) was performed to determine the spectral changes of CB (Gould & Vosburgh, 1942). Separate solutions of 30 μg/ml CB and TMC were prepared in phosphate buffer (pH 7.4). These solutions were added to each other in 11 test tubes with increasing volumes of TMC (0–10 ml) and decreasing volumes of CB (10–0 ml). The test tubes were centrifuged and the spectrophotometric absorbance of supernatant was determined at 578.6 nm for determination of free amount of CB. In order to check whether the changes in the spectrophotometric absorbance is due to pH changes upon addition of TMC, pH of the test tubes were determined.

2.9. Statistical analysis

Based on the 2^3 full factorial design (Table 1), linear multiple regression models for the data were calculated. After constructing these models, assumptions underlying these tests, such as absence of co-linearity, homogeneity of variance, as well as normality of the data were tested and found to be met. *P* value of less than 0.05 was considered as significant. Statistical analysis of the results for all eight formulations was performed to determine the best formulation. SPSS 11.0 software for Windows was used for statistical analysis.

3. Results and discussion

3.1. Synthesis and characterization of TMC

TMC was successfully synthesized by the two-step method. NMR spectrum of TMC showed 65% degree of quaternization (Fig. 1). The synthesized TMC was used in the preparation of beads.

3.2. Rationale for selection of ingredients and processes

Beads prepared with PLMA concentrations less than 3.5% had a weak consistency and shrank significantly upon drying and lost their spherical shape and became somewhat flat.

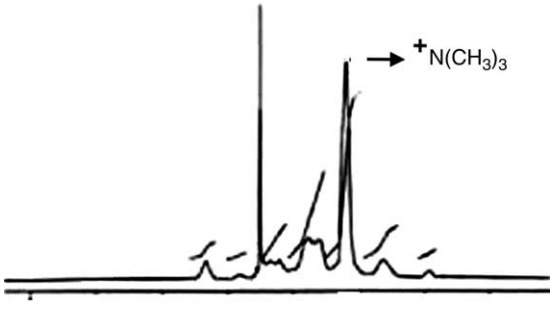

Fig. 1. ^{1}H NMR spectrum of *N*-trimethyl chitosan chloride. The peak at 3.4 ppm (shown by arrow) denotes the $^+$N $(CH_3)_3$ groups.

On the other hand, pectin concentration of more than 5% produced a very viscose gel and the beads prepared with it had an undesirable drop-like shape; thus in the factorial design final concentrations of 3.5 or 5% were chosen for PLMA.

Based on the following preliminary studies, Zn acetate was used as an alternative cross-linker to Ca chloride. Wellner et al. (1998) assessed pectinate gels (not beads), formed by various divalent cations and compared their physical state. In their study it is stated that the size and charge density of ions are important parameters in the gel properties. They observed that Zn^{2+} forms gels even with pectin with degree of methoxylation of 59.1% (where high ester groups eliminate the negative charge on pectin chain and may also sterically hinder the formation of chain aggregates), while Ca^{2+} does not; therefore Zn^{2+} is a stronger cross-linker than Ca^{2+}. Our studies confirmed this observation as well. Beads cross-linked by Ca chloride had a slightly lower loading efficiency compared to those cross-linked by Zn acetate (Table 2, formulations Fa and Fb). The effect of cross-linker type (Zn acetate or Ca chloride) on the release behavior and SER of beads is shown in Fig. 2. As seen in Fig. 2a and b, Fa releases CB much faster than Fb and it has completely eroded in 1.5 h in phosphate buffer (Fig. 2c).

With regards to the safety of application of Zn acetate, the weight of the pectinate beads (without TMC or CB), was compared to the weight of the dry content of pectin in the initial gel volume (350 or 500 mg/10 ml, respectively). The weight of the beads was the same as the weight of the dry content of pectin. Thus it can be concluded that the amount of Zn^{2+} in the beads is less than 1 mg/350 or 500 mg bead (possible daily dosage of the beads). Zinc is an essential micronutrient with recommended dietary allowance (RDA) of 10 mg (NIH's Office of Dietary Supplements, 2002). Rats ingesting up to 25.5 mg Zn/kg/day (as zinc acetate) in drinking water for 47 weeks exhibited no overt toxic effects (Drinker, Thompson, & Marsh, 1927). Therefore, the amount of less than 1 mg Zn^{2+} in the beads is surely safe. Consequently, Zn acetate was chosen as the cross-linker for optimization of beads.

Beads were cross-linked with Zn acetate for 2, 4, or 24 h to determine the effect of t_c on beads' characteristics (Fig. 3a–c). The release characteristics and SER of these

Table 2
Loading efficiency (LE) of pectinate beads prepared in the preliminary studies

Formulation Code	Cross-linker type	TMC:PLMA ratio (w/w)	t_c (h)	LECB (%)
Fa	Ca	1:6	2	99.2
Fb	Zn	1:6	2	100
Fc	Zn	1:6	4	100
Fd	Zn	1:6	24	100
Fe	Zn	–	2	95.9
Ff	Zn	1:8	2	99.9
Fg	Zn	1:4	2	100

PLMA concentration = 3.5% (w/v), cross-linker concentration = 0.15 M, nozzle diameter = 1.1 mm, encapsulator voltage = 2 kV.

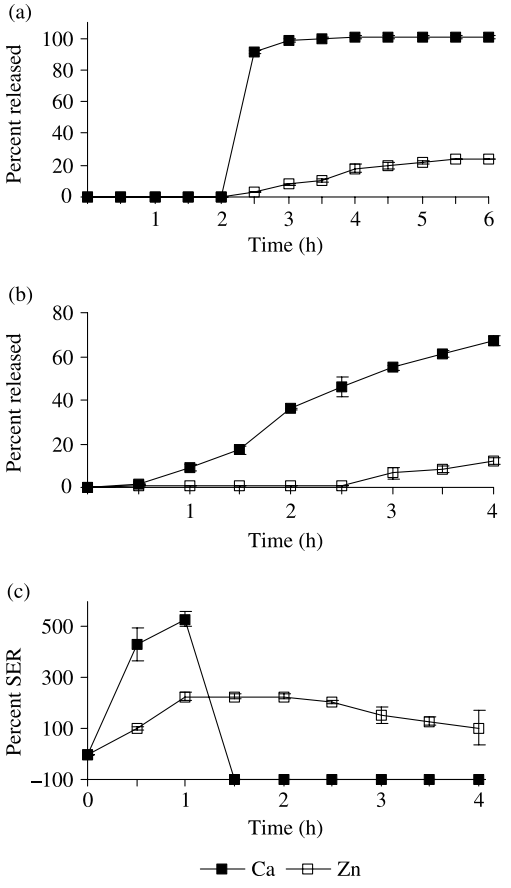

Fig. 2. Influence of cross-linker type on beads' characteristics. In vitro behavior of TMC–pectinate beads cross-linked either with Zn acetate or Ca chloride (mean ± S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling–erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLMA concentration = 3.5%, crosslinker concentration = 0.15 M, $t_c = 2$ h, TMC:PLMA ratio = 1:6, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm.

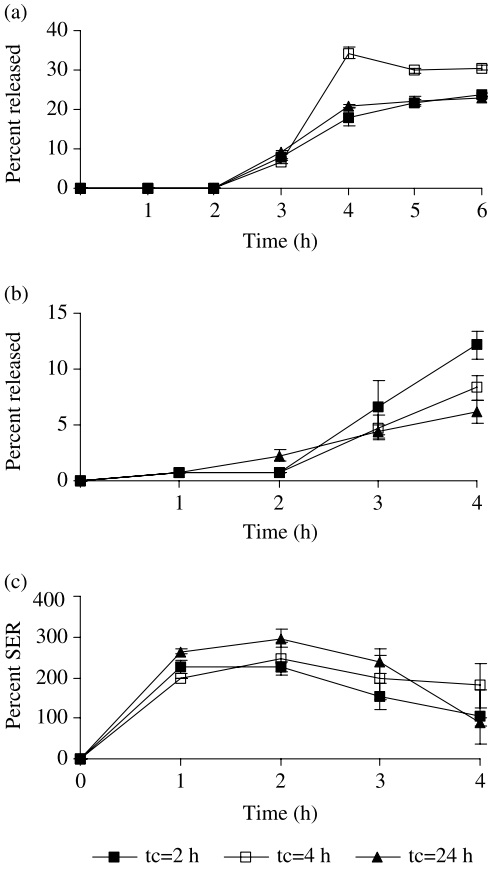

Fig. 3. Influence of cross-linking time (t_c) on beads' characteristics. In vitro behavior of TMC–pectinate beads cross-linked with Zn acetate either for 2, 4 or 24 h (mean ± S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling–erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLMA concentration = 3.5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm.

formulations show that there is no significant difference between the release behavior of beads cross-linked for 2, 4 and 24 h. Thus t_c of 2 h was selected.

TMC is said to be an effective absorption enhancer at concentrations of 0.5–1% (w/v) in the absorption site (Hamman, Schultz, & Kotzé, 2003; Jonker, Hamman, & Kotze, 2002). Therefore, it was included in the formulations. The higher the concentration of TMC in a formulation, the lower is the amount of the formulation that has to be administered, for the absorption enhancer to be in the effective range. However, excessive amounts of TMC in the formulations led to impaired beads characteristics (Fig. 4a–c); Therefore, TMC concentration was fixed at the optimum level of 1:6 of PLMA concentration.

3.3. Experimental design and optimization of beads

Zn pectinate beads were prepared based on the 2^3 full factorial design (Table 1). The loading efficiency of these formulations for both, CB and TMC was 100%. The release studies were carried out with these beads in simulated gastrointestinal conditions (Fig. 5a and b). The SER studies were also performed on these beads (Fig. 5c). From the results of Table 1, the following linear regression models were derived Eqs. (6) and (7):

$$\begin{aligned}\mathrm{Rel}_{4h} &= 32.506(\pm 8.218) - 5.4(\pm 1.904)\mathrm{CPLMA} \\ &\quad + 84.444(\pm 18.792)\mathrm{CZnacetate} \\ &\quad - 17.407(\pm 4.354)\mathrm{CPLMA} \times \mathrm{CZnacetate} \\ P\,\text{value}&: (0.001)\ \ (0.01)\ \ (0.000)\ \ (0.001)\ \ \mathrm{AR}^2 = 88.2\%\end{aligned} \tag{6}$$

$$\begin{aligned}\mathrm{SER}_{4h} &= 30.556(\pm 13.442)\mathrm{CPLMA} \\ &\quad - 420.370(\pm 44.806)\mathrm{CZnacetate} \\ &\quad - 24.583(\pm 10.081)\mathrm{Voltage} \\ P\,\text{value}&: (0.034)\ \ (0.000)\ \ (0.024)\ \ \mathrm{AR}^2 = 77\%\end{aligned} \tag{7}$$

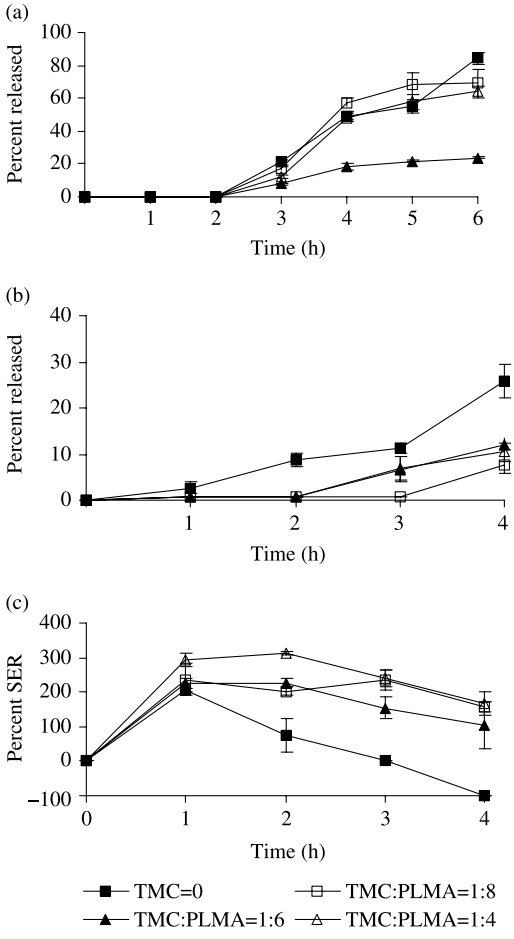

Fig. 4. Influence of TMC:PLMA ratio on beads' characteristics. In vitro behavior of TMC–pectinate beads with TMC-PLMA ratio of 1:8, 1:6, 1:4 (mean ± S.D., $n=3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling–erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLMA concentration = 3.5%, Zn acetate concentration = 0.15 M, $t_c=2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm.

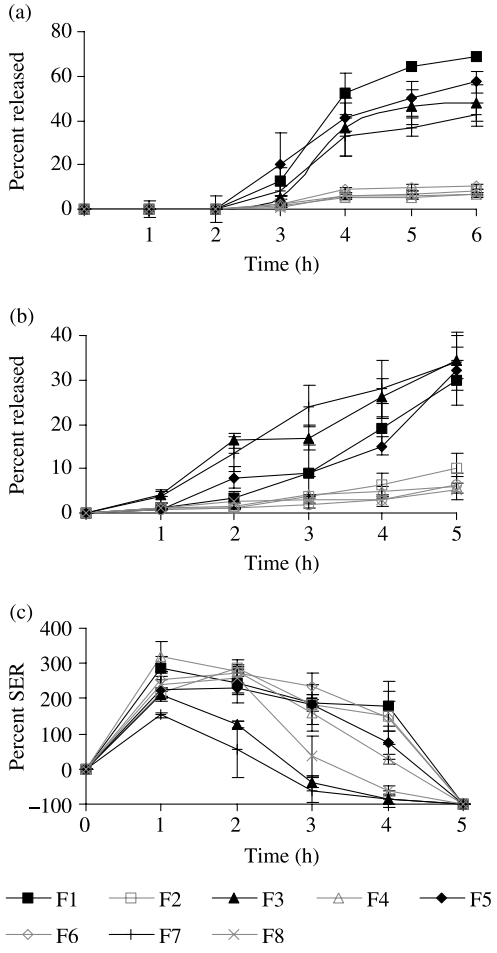

Fig. 5. Effect of PLMA concentration, Zn acetate concentration and encapsulator voltage on beads' characteristics. In vitro behavior of TMC–pectinate beads prepared based on the 2^3 factorial design (Table 1) (mean ± S.D., $n=3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (5 h). (c) swelling–erosion ratio (S.E.R.) of beads in phosphate buffer pH 7.4 (5 h). $t_c=2$ h, TMC:PLMA ratio = 1:6, nozzle diameter = 0.7 mm.

Rel_{4h} is cumulative percent of CB released in 4 h from beads in phosphate buffer (pH 7.4) and SER_{4h} is SER of beads in 4th hour in phosphate buffer (pH 7.4). CPLMA is PLMA concentration in the beads (% w/v). CZnacetate is concentration of Zn acetate in cross-linking solution (mM). Voltage is voltage of the bead generator (kV). The P value of each term in the equations is subscribed under the term.

As clearly seen in Fig. 5a and b, and based on the results of Eqs. (6) and (7), PLMA concentration has a dramatic effect on beads' characteristics. Higher PLMA concentrations in the beads, lead to formation of denser matrix, which improves the beads' release behavior and retards the CB release from the beads. Moreover, we can deduce from Eq. (7) that beads with higher PLMA, are favored with regards to preserving their multi-particulate structure.

We can conclude from the Eqs. (6) and (7) and Fig. 5, that release and swelling–erosion behavior of beads are dependant on Zn acetate concentrations. Excessive Zn acetate increases the CB release, in formulations with lower PLMA concentration. On the other hand, it causes an early phase of swelling, followed by rapid erosion of the beads. We used high concentrations of Zn acetate to obtain higher cross-linking of the PLMA matrix, but on the contrary, in case of beads with lower PLMA concentration, the beads' characteristics were impaired. This observation can be explained by the fact that Zn acetate is a slightly acidic salt (pH of 5% solution is 6). Therefore, its excessive application as a cross-linker might tend to transform some of COO^- groups of PLMA into COOH groups (which are unavailable for cross-linking). Moreover, once the dried beads are placed in the aqueous media, high Zn acetate concentration entrapped in the beads exerts an osmosis phenomenon and absorbs the water into the beads. Thus the swelling of the beads is accompanied by erosion and drug release. On the other hand, the interaction of Zn acetate

concentration and PLMA concentration is also observed to be significant in Eq. (6). In case of formulations with higher PLMA concentration, increasing Zn acetate concentration causes a very slight decrease in drug release (Table 1). However, this decrease is infinitesimal and within the standard deviation range of the data and not statistically significant; therefore in these formulations, Zn acetate concentration seems not to be influential in bead's release behavior. This might be due to the dense pectin matrix of the beads with higher PLMA concentration, which overshadows the above-stated adverse effects of excessive Zn acetate concentration on release. Consequently, since excessive Zn concentration either has a negative or no effect on bead characteristics, it should be used in optimized concentrations to render enough cross-linking to the beads, yet its unwarranted high concentrations should be avoided in bead preparation.

Voltage of the bead generator did not have any effect on the Rel_{4h} of the beads Eq. (6); however, it inversely affected the SER_{4h} of the beads Eq. (7). Nevertheless the regression coefficient for voltage (−24.583) is trivial compared to the coefficient of Zn acetate concentration (−420.370). Moreover, the *P* value of this independent variable is 0.037, which is higher than the *P* value of Zn acetate concentration. Therefore the effect of encapsulator voltage is less pronounced than the effect of Zn acetate concentration. Consequently based on the results of Eqs. (6) and (7), and Fig. 5, it was concluded that the beads can be prepared with electrostatic bead generator without impaired characteristics.

Based on the overall results of the one-way analysis of variance (ANOVA) and multiple comparison (Duncan's test), for release and SER of the F1–8 formulations, F6 was chosen as the optimized core formulation. Moreover, nozzle diameter of 1.1 mm was chosen for further experiments, because beads prepared with 1.1 mm nozzle had better sphere-like shape and were easier to coat.

Although F1–8 formulations showed release of 20–30% in phosphate buffer (pH 7.4) after 5 h, by referring to the swelling–erosion data, it is noted that their SER is declining, and consequently in 5 h, SER is −100% for all formulations. This low percent of release (20–30%), might seem perplexing, when at the same time SER value is −100%. In this regard, one should bear in mind that a SER value of −100% does not necessarily mean that the beads have completely dissolved. In some cases SER value of −100%, shows that beads are no longer in the multi-particulate from (see Section 2.6); thus it is possible (as macroscopically observed by us in such cases), that beads keep some of their drug unreleased. Actually, in such cases the beads turned into a blue shapeless gel, which retained some of the drug. Besides, it is possible that some of the CB in the beads, form a complex of decreased solubility, with TMC (see Sections 2.8. and 3.6.). This complexation modifies the ultimate drug release from beads. Therefore, the part of CB, entrapped in the insoluble complex with TMC, is in the form of a precipitate and is not included in the withdrawn samples, and cannot be detected by spectrophotometer.

These experiments show that although the release data alone seem satisfactory, the beads could not retain their spherical and multi-particulate structure. Therefore, they could not fulfill our goals of colon targeting along with maintaining their multi-particulate form. Hence, the beads had to be further modified

3.4. Rationale for modification of beads

The release and swelling–erosion data of the beads, in all our experiments showed that while no drug release and swelling occurs in 2 h of acid exposure (pH 1.5), pre-exposure to acidic medium drastically affects the beads' behavior and decreases the resistance in phosphate buffer (pH 7.4). The possible explanation of this phenomenon is that in the acidic medium the carboxylic group of pectin in the surface of the beads, turns into COOH, because the pH of the medium (1.5) is less than the pK_a of pectin (3.5) (CP Kelco information sheet); Therefore, at this pH, due to less hydrophilicity and lack of repulsion between carboxylic groups, Zn pectinate (or even uncross-linked pectin) is in the form of a relatively insoluble and tightly packed gel. Thus no swelling and release is observed. At the same time, at this pH, displacement of Zn^{2+} with H^+ or K^+ occurs in the outer layers of beads via diffusion. This way the egg-box structure is depleted of its cross-linking agent, but since pectin is insoluble in acidic environment the beads keep their spherical structure in macroscopic observation. Thereafter upon change of medium and entrance into pH 7.4, the COOH groups once again turn into COO^-, which repel each other. Since some cross-linker is depleted from the beads, the structure is loose and a dramatic swelling and subsequent erosion and drug release occurs. These findings compelled us to modify the beads to obtain the desired profiles.

A conventional way to protect the beads from exposure to harsh gastric environment is to put them into capsules or shape them into a tablet form and enteric-coat the single unit dosage form. Nevertheless by the conventional method stated above, the advantages of the multiple-unit dosage form are lost (see Section 1). The gastric emptying time is the most important source of variation in the gastrointestinal transit time. The multiple-unit dosage forms act reproducibly and predictively in this regard. Therefore, in this study it was imperative to keep the multiple-unit dosage forms throughout the modification experiments. These beads were prepared by 1.1-mm nozzle because with this nozzle they were more spherical in shape and the coating of the beads were easier.

3.5. Characterization of modified pectinate beads

3.5.1. Characteristics of biomineralized beads

Biomineralization was not a successful approach for improvement of beads, and it even worsened the beads'

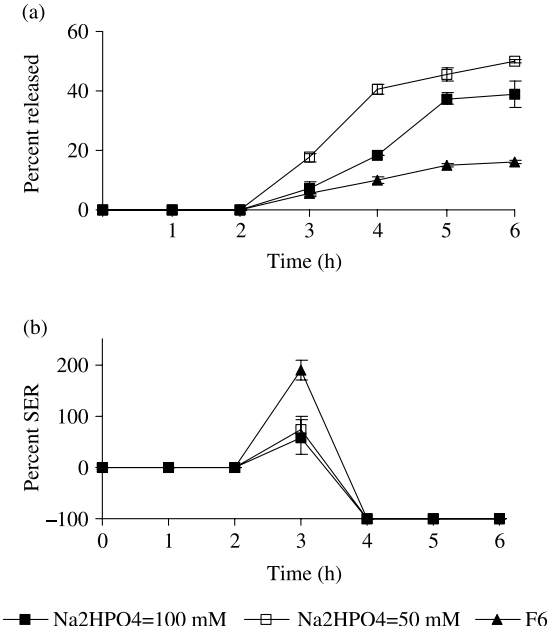

Fig. 6. In vitro behavior of biomineralized TMC–pectinate beads (mean ± S.D., $n=3$). (a) release profile of CB from biomineralized beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) swelling–erosion ratio (SER) of biomineralized beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). Based on F6 formulation of Table 1; PLMA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, $t_c = 2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm Na_2HPO_4 concentration = 50 or 100 mM.

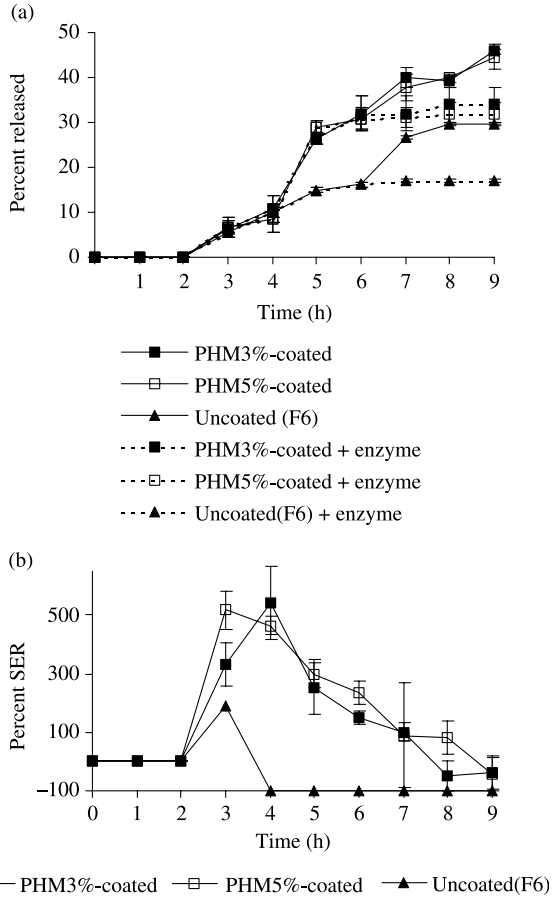

Fig. 7. In vitro behavior of PHM-coated TMC–pectinate beads (mean ± S.D., $n=3$). (a) release profile of CB from PHM-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h) and phthalate buffer pH 5 with/without 50 μl of Pectinex Ultra SPL (3 h). (b) swelling–erosion ratio (SER) of PHM-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). The core formulation is F6 formulation of Table 1. PLMA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, $t_c = 2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm. PHM concentration = 3 or 5%.

behavior (Fig. 6a and b). This may be due to the displacement of some of Zn^{2+} by Na^+ from Na_2HPO_4. Sodium pectinate and other monovalent salts of pectin are in the form of a non-viscous liquid and do not form gel at neutral environment (Wellner et al., 1998). Moreover, the discrepancy between our results and the results obtained by Leveque et al. (2002), might arise from the fact that in the latter study, the release profile of the beads was tested in deionized water. This can affect the results in two ways: (a) pH value of deionized water is 6. At this pH value the solubility of pectin is lower than in pH 7.4. (b) As there is no ion present in deionized water, no sequestration or displacement of crosslinker occurs between the beads and the medium.

3.5.2. Characteristics of PHM-coated beads

The results of release studies on PHM-coated beads show somewhat higher release (yet below 30%), compared to uncoated beads. However, the SER data demonstrates that despite this higher release, the PHM coat is capable of keeping the integrity of beads, while the uncoated beads loose their structure within 4 h (2 h in acidic medium and a successive 2 h in phosphate buffer, pH 7.4) (Fig. 7a and b). The higher CB release from PHM-coated beads might be due to prolonged exposure of the core to a PHM layer of local moist viscous gel during the coating process and also in release environment, which withdraws some of the drug to the surface, and upon erosion and dissolution of the PHM coat, creates a burst-like release. Upon the change of medium to phthalate buffer (pH 5) with Pectinex Ultra SPL, PHM-coated beads crumbled to solid pieces and the SER could not be further determined. Surprisingly, in the presence of enzyme, no further CB release was observed both for F6 and PHM-coated formulations. On the other hand, in the absence of pectinolytic enzyme, the PHM-coated beads were initially in the form of gel-like beads, which completely eroded within 3 h and continued to release CB.

With regards to the paradoxical phenomenon observed with the pectinolytic enzyme, it was doubted that the enzyme might reduce the solubility of CB and impair its release from the beads. However, this hypothesis was ruled out by determining the spectrophotometric absorption of CB in solutions of known concentration, in phthalate buffer, either with or without the enzyme. The enzyme had no effect

on the solubility of CB. From the macroscopic inspection of the beads exposed to the pectinolytic enzyme, it appeared that the pectinolytic enzyme, suppresses further water-uptake of the beads (possibly by hydrolysis of glycosidic linkages, breaking the polymer chain, and reducing its gel-forming properties). Less water-uptake of the beads might be partially implicated in the inhibition of CB release from pectinate beads. Such results are also reported by Semdé, Amighi, Devleeschouwer, & Moës (2000). They observed that pectinolytic enzymes slowed down the theophylline release from the pellets coated with ethylcellulose or polymethacrylate polymers, containing 10% w/w of PHM or calcium pectinate. They proposed that in the presence of pectinolytic enzymes, pectin is rapidly degraded and leached out of the film coating, therefore the swelling and the hydration of the film-coatings is decreased. Moreover, the film-coating restructures and plugs up the pores (formed by leaching of pectin), slowing down the drug release. On the other hand, in the absence of pectinolytic enzyme, pectin rapidly absorbs the surrounding water, since PHM and particularly calcium pectinate are hydrophilic and good gel-forming polymers. The pectin, which is now hydrated, can swell and induce a further increase of the swelling and the hydration of the film-coatings, the formation of hydrated pectin channels and probably, the appearance of distensions in the film-coatings. As a result, the diffusion of hydrophilic drugs such as theophylline through the film-coatings is improved. Such mechanisms might be involved in our system as well.

Nonetheless, the colonic pectinolytic enzymes may act differently, in vivo. Moreover, as discussed earlier (see Section 2.5.), we chose a pH value of 5 for colonic medium, to optimize the enzymatic activity. This issue may weaken the relevance to the colonic environment, in vivo, and the above argument might be only of academic value. Therefore, further studies either in vivo, or with rat cecal content should be carried out to clarify the actual effect of pectinolytic enzymes on the beads behavior.

3.5.3. Characterization of EU-coated beads

EU-coated beads exhibited satisfactory characteristics with regards to release and swelling–erosion studies. The optimized coating process ensures that a flawless coat encloses the beads, which is impenetrable to acidic medium. The coat dissolves within one hour in phosphate buffer (pH 7.4), releases the cores in intestinal medium in intact state (Fig. 8a and b), which will then reach colon. As observed for PHM-coated beads, when EU-coated beads, (whose EU coat was then completely dissolved), were transferred to phthalate buffer (pH 5) with Pectinex Ultra SPL, they crumbled and their SER could not be determined. In the same way, no further CB release from these formulations, occurred in the presence of enzyme. However, in the phthalate buffer (pH 5) alone, EU-coated beads started to swell and release the model drug.

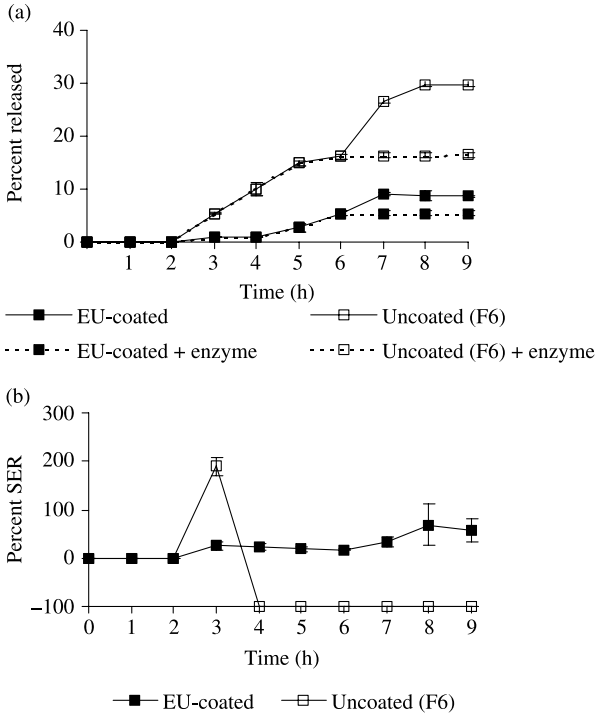

Fig. 8. In vitro behavior of EU-coated TMC–pectinate beads (mean ± S.D., $n = 3$). (a) release profile of CB from EU-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h) and phthalate buffer pH 5 with or without 50 μl of Pectinex Ultra SPL (3 h). (b) swelling–erosion ratio (SER) of EU-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). The core formulation is F6 formulation of Table 1. PLMA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, $t_c = 2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm.

The dependence of PHM- and EU-coated pectinate beads on microbially triggered mechanisms, should be further studied. However, apart from the microbially triggered mechanisms, these systems appear to be time- and pH-controlled.

3.6. CB-TMC complexation

Comparison of Fe, Ff, Fb and Fg formulations (TMC:PLMA ratio of 0, 1:8, 1:6 and 1:4, respectively), show that increasing amounts of TMC in formulations led to slightly higher (although not significant) loading efficiency of beads for CB (Table 2). Fig. 3 also illustrates that

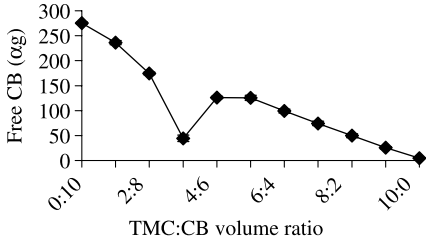

Fig. 9. Job's plot of CB-TMC in phosphate buffer pH 7.4, investigated by spectrophotometric absorption of CB at 578.6 nm (mean ± S.D., $n = 3$).

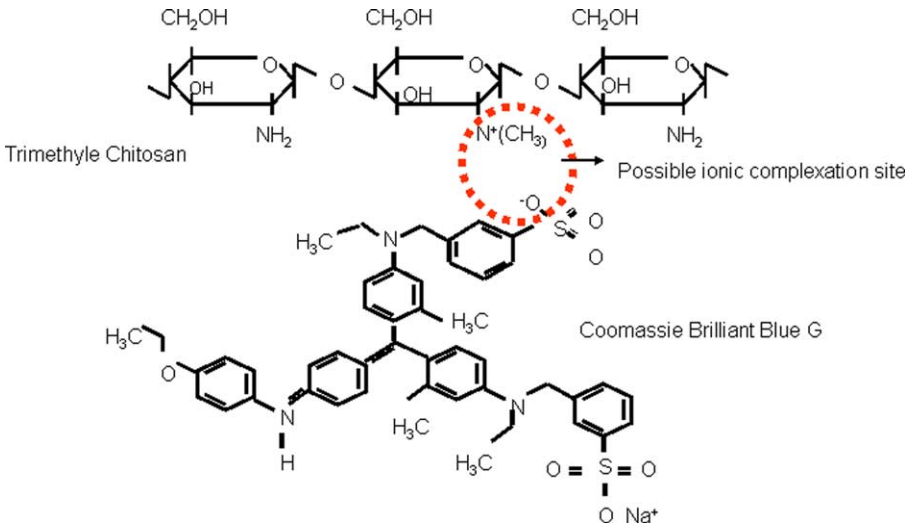

Fig. 10. Structure of trimethyl chitosan and Coomassie Brilliant Blue G 250 and their possible ionic complexation.

the increase in TMC amount modifies CB release and SER of beads.

Fig. 9 illustrates the Job's plot for CB-TMC. Upon increasing TMC:CB volume ratio, (which in fact corresponds to increasing TMC:CB weight ratio), at some point a steep decrease in the free CB concentration is observed, which does not conform to the rest of the curve. This point (TMC:CB = 2:3) denotes the optimum weight ratio of complex formation between CB and TMC. In macroscopic inspection, at this point a blue precipitate was observed in the test tube. The pH values of the test tubes were measured and it was observed that the values had not changed from that of the diluting buffer (pH 7.4). Therefore, the possibility that changes in pH, upon addition of TMC, might have caused this phenomenon, was ruled out. This interesting finding can be exploited to inhibit premature release of drugs in any dosage form, including the beads prepared in this study. Some studies have proposed that the ionic interactions can be used for controlled delivery and release of drugs. Bernkop-Schnürch, Schuhbauer, Clausen, and Hanel (2004), developed a sustained-release dosage form for α-lipoic acid based on ionic interactions between the cationic polymer chitosan and the anionic drug. Shalaby, Jackson, and Moreau (2001) invented a sustained release composition, which features polyester containing free COOH groups ionically conjugated with a biologically active polypeptide composed of at least one effective, ionogenic amine.

CB is reported to have three pK_as with values of 1.15 and 1.82 and 12.4, with, respectively, red, green and blue colors (Chial, Thompson, & Splittgerber, 1993). At the pH of PLMA gel mixture (pH ~ 4), CB is in the blue color and it is in the deprotonated form. Thus the $^{+}N(CH_3)_3$ groups of TMC form ionic bonds with SO_3^- moieties on the CB (Fig. 10). Nevertheless, the formulations, which contain TMC, do not release 100% of their loaded CB in the soluble form, even after the beads are completely degraded. Therefore, CB-TMC complex may alter the final amount of released drug from the dosage forms, or mask the absorption-enhancing effect of TMC. Therefore, further in-depth investigation is required to elucidate this phenomenon and assess its feasibility.

4. Conclusion

Pectinate-TMC beads can be promising, multi-particulate dosage forms for colon-specific drug delivery. However, they should be protected from gastric acidic environment. The optimized system was designed by coating the beads with either PHM or EU. In vitro studies confirmed that this system is capable of delivering the water-soluble high molecular weight drug together with TMC as an absorption enhancer, to colon. This multiparticulate system appears to be both, time- and pH-controlled and possibly microbially triggered. Further studies are underway to assess the in vivo feasibility of these systems for peptide and protein drugs.

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