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Colon-specific delivery of resveratrol: Optimization of multi-particulate calcium-pectinate carrier

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ABSTRACT

This study aimed at devising multi-particulate calcium-pectinate (Ca-pectinate) bead formulations for colon-targeted delivery of resveratrol. As Ca-pectinate beads were not sufficient to endure the upper GI environment and premature release of resveratrol occurred before their arrival to the colon, the beads were hardened by adding polyethyleneimine (PEI) in the cross-linking solution. The effects of PEI concentration, cross-linking time, and pectin to resveratrol ratio were investigated on beads' characteristics, encapsulation efficiency, swelling–erosion, and resveratrol retention pattern of formulated beads. Proper conditions were optimized from these studies and optimized beads were further subjected to morphological examination. Formulated beads were spherical with ~1 mm diameter. Addition of PEI to the cross-linking solution and a minimum cross-linking time were found to be crucial factors for colon-specific release of resveratrol. As PEI was added in the cross-linking solution, hardening of the bead surface occurred simultaneously with bead formation. Observations from the present study revealed that optimized Ca-pectinate beads hardened with PEI can efficiently encapsulate resveratrol and have potential for colon-specific delivery to the lower GI tract.

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a polyphenolic phytoalexin, exhibits several pharmacological activities, such as, anti-oxidant, anti-inflammatory, analgesic, cardio-protective, neuro-protective, chemo-preventive, anti-aging etc (Jang et al., 1997; Baur and Sinclair, 2006). In addition, resveratrol has shown promising therapeutic efficacy towards several lower gastro-intestinal (GI) tract diseases like, colon cancer and colitis (Tessitore et al., 2000; Schneider et al., 2001; Martin et al., 2004, 2006). Together with pharmacological activities, its pharmacokinetics has also been investigated in pre-clinical and clinical models (Baur and Sinclair, 2006; Delmas et al., 2006; Das et al., 2008). In spite of rapid absorption through the GI tract, resveratrol is quickly metabolized in the upper GI tract and liver following oral administration (Marier et al., 2002; Gescher and Steward, 2003; Kaldas et al., 2003; Baur and Sinclair, 2006; Maier-Salamon et al., 2006). Rapid declination of plasma resveratrol concentration and very low oral bioavailability was evident in our previous study (Das et al., 2008). The poor oral bioavailability and extremely short plasma half-life of resveratrol have raised concerns regarding its systemic action. Furthermore, because of the rapid absorption through the upper GI tract, insufficient amount of resveratrol will reach to the colonic region for the treatment of the colon diseases (Das et al., 2008). Therefore, we hypothesized that a colon-specific delivery system of resveratrol might be a better alternative to the existing treatment of the lower GI tract diseases, such as, colorectal cancer and colitis. This kind of delivery systems protect drug during its transit through the upper GI tract and allow its release at the colonic region, which should be able to bypass the rapid absorption and metabolism issues of resveratrol.

Currently, four types of colon-specific drug delivery systems are exploited. Those are the systems that are controlled by GI transit time, GI pressure differences, pH differences of GI tract, and colonic bacterial enzymes (Basit, 2005; Friend, 2005). However, a drug delivery system, which will be intact at upper GI conditions and specifically degraded in contact with the bacterial enzymes that are predominantly present in the colon, are more realistic approach than the other approaches due to less inter-individual variability (Liu et al., 2003; Basit, 2005).

In this ambit, pectins (natural polysaccharides) appeared to be suitable carriers for colon-specific delivery system due to their specific degradation by the colonic bacterial enzyme when resis-

Abbreviations: PEI, polyethyleneimine; GI, gastro-intestinal; $CaCl_2$, calcium chloride; Ca-pectinate, calcium pectinate; SER, swelling–erosion ratio; EE, encapsulation efficiency; MC, moisture content; WL, weight loss during drying; Ca, calcium; Ca^{2+}, calcium ion; ER, elongation ratio; L, resveratrol loading; RT, room temperature; P:R, pectin to resveratrol ratio.

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tant to the enzymes present in the stomach and intestine (Friend, 2005). They are composed of partially methoxylated polyα (1-4)-D-galacturonic acids with some 1–2 linked L-rhamnose groups and present in the cell wall of most of the edible plants (Liu et al., 2003). Oral intake of them is proved to be safe. Although their solubility and swellability in the basic/neutral fluids (e.g., intestinal fluid) prevent them to be used as efficient colon-specific drug carriers, their combination with other additives may solve this problem (Liu et al., 2003). Pectins with low degrees of methoxylation (i.e., low methoxy pectins or LM pectins) can be cross-linked with calcium ions (Ca^{2+}, divalent cations) and produce Ca-pectinate networks that are more water insoluble (Sriamornsak and Nunthanid, 1998, 1999; Bourgeois et al., 2006). Various reports suggest that these Ca-pectinate networks have the potential to be an effective vehicle for drug delivery (Sriamornsak, 1999; Maestrelli et al., 2008a,b). Moreover, multiple unit dosage forms proved to be better than single unit dosage forms due to their reproducible and predictable GI transit time, more reliable drug release profile, and less local irritation (Maestrelli et al., 2008a).

In fact, we have developed multi-particulate Ca-pectinate bead formulation of resveratrol in our previous study (Das and Ng, in press). To our knowledge, so far that was the only study which looks at the delivery of resveratrol specifically to the colonic region. However, similar to findings by others (Sriamornsak, 1999), we found that pectinate bead alone was insufficient in protecting resveratrol release at the small intestinal pH, although it prevented resveratrol release at the acidic pH of stomach (Das and Ng, in press). Modification of Ca-pectinate bead is therefore needed to achieve colon-specific delivery of resveratrol. Recently, PEI has been evaluated as a hardening agent for the pectin formulations (Bourgeois et al., 2005, 2008). Hence, in the present study, we have formulated resveratrol-loaded Ca-pectinate beads hardened with PEI and subsequently evaluated the effects of PEI concentration, hardening time, and pectin to resveratrol ratio on the beads' characteristics and resveratrol release profiles. We have optimized the formulation parameters mainly based on resveratrol retention patterns in simulated GI conditions and resveratrol encapsulation efficiency of the beads.

2. Materials and methods

2.1. Materials

GENU® pectin type LM-104 AS-FS (amidated LM pectin; degree of esterification (DE) = 28% and degree of amidation (DA) = 20%) was a generous gift from CPKelco (Denmark). Calcium chloride dihydrate and resveratrol (99.12% purity, fine crystalline powder) were purchased from Merck (Darmstadt, Germany) and Shaanxi Sciphar Biotechnology Co., Ltd. (Xi'an, China), respectively. Polyethyleneimine (PEI), Pectinex® Ultra SP-L (pectinase/pectinolytic enzyme from *Aspergillus aculeatus*), sodium hydroxide, and sodium phosphate monobasic were purchased from Sigma (St Louis, MO, USA). HPLC grade methanol (Tedia Company, Fairfield, OH, USA), purified water (18.2 MΩ cm at 25 °C from Millipore Direct-Q® ultra-pure water system, Billerica, MA, USA) were used throughout the study. Monobasic potassium phosphate and di-sodium hydrogen phosphate anhydrous were obtained from Fluka (Steinheim, Germany). All materials were used as received.

2.2. Formulation

Ca-pectinate beads were prepared using a method (ionotropic gelation) modified from our previous study (Fig. 1) (Das and Ng, in press). Briefly, pectin (5%, w/v) was dissolved in deionized water and resveratrol was homogeneously dispersed in it by a homogenizer. Air bubbles were removed from the dispersion by sonication on a bath sonicator. The pectin–resveratrol mixture was then added dropwise (1 ml min^{-1} from 5 cm distance) to a gently agitated cross-linking solution (10% calcium chloride aqueous solution (pH 5.5) containing different concentration of PEI) through a blunt end needle (25 G) at room temperature (RT). The beads formed were allowed to stand in the solution for specified time interval with gentle agitation. The beads were then separated, washed with distilled water, and subsequently dried at RT for 24 h.

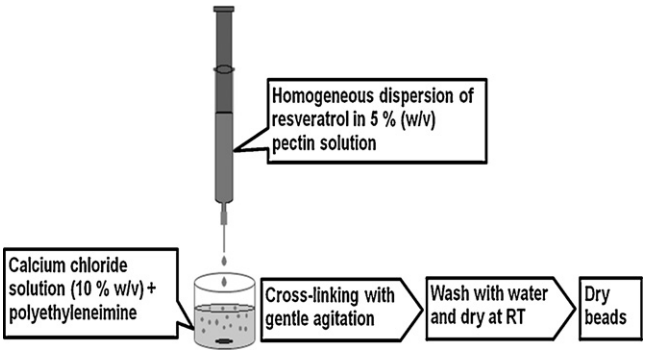

Fig. 1. Schematic presentation of the formulation procedure.

The beads were prepared by varying the formulation variables (PEI concentration, cross-linking time, and pectin to resveratrol ratio) listed in Table 1. The effects of different formulation variables on beads' size, shape, moisture content (MC), water loss during drying (WL), resveratrol loading (L) and encapsulation efficiency (EE), swelling and erosion behavior, and resveratrol retention patterns in simulated GI conditions were thoroughly evaluated. The formulation variables were optimized after observing their effects on beads' characteristics, EE, and resveratrol retention profile in simulated GI conditions. All batches were prepared in triplicate.

2.3. Morphology

The micrographs of surface and cross section of the resveratrol-loaded beads hardened with PEI were recorded using a JEOL scanning electron microscopy (JSM-5200) at an excitation voltage of 20 kV. Pectinate beads were fixed on an aluminum stub and coated with platinum for 30 s (coating thickness ~2 nm) under vacuum by auto fine coater (JFC—1600 (JEOL)) and images were recorded at different magnifications. Furthermore, the beads' surface was compared with the surface of the non-hardened beads and resveratrol-free beads.

2.4. Size and shape

The size and shape of the wet and dry beads were measured by an optical microscope (LEICA DM IL, Switzerland). Fifty beads from each batch were randomly selected for the study. The length and breadth of each bead was measured by the pre-calibrated image analysis program (LAS EZ, version 1.4.0) after images were captured through a digital camera (LEICA EC3, Switzerland) connected with the microscope. The size of each bead was calculated from the average of these two dimensions (Wong and Nurjaya, 2008; Das and Ng, in press). The average size of the beads from each batch was expressed as the mean diameter (μm) ± standard deviation (SD).

The shape of the above mentioned beads was represented as elongation ratio (ER) which is the quotient of length to breadth of the beads (Wong and Nurjaya, 2008; Das and Ng, in press). ER = 1, 1 < ER < 1.15, and ER > 1.15 represent a perfect spherical, spherical, and non-spherical shape, respectively. The mean diameters

Table 1
Formulation design.

Variables	Values	Constants		
		PEI concentration (% w/v)	Cross-linking time (h)	Pectin:resveratrol
PEI concentration (% w/v)	0	–	2	3:1
	0.05	–	2	3:1
	0.1	–	2	3:1
	0.2	–	2	3:1
	0.3	–	2	3:1
Cross-linking time (h)	0.08	0.2	–	3:1
	0.5	0.2	–	3:1
	2	0.2	–	3:1
	24	0.2	–	3:1
Pectin:resveratrol	1:1	0.2	2	–
	3:1	0.2	2	–

and shapes of the beads before and after drying were compared.

2.5. Weight loss during drying

Gravimetric analysis was performed to determine the WL of the beads (Das and Ng, in press). Randomly selected 50 beads from each batch were weighed with an analytical balance with readability of 0.00001 g (Metler Toledo, Switzerland) before and after drying. The average weight loss (WL) was calculated by the following equation:

$$\mathrm{WL}(\%) = \frac{W_W - W_D}{W_W} \times 100\% \quad (1)$$

where W_W is the weight of the beads measured just after washing and W_D is the weight of the dry beads. Experiment was performed in triplicate for each batch and the average WL was expressed as the mean WL (%) ± standard deviation (SD).

2.6. Moisture content

MC of the beads was also determined using gravimetric method (Das and Ng, in press). Fifty randomly selected beads from each batch were dried completely at elevated temperature (60 °C) until no further weight change was observed. Moisture content was calculated using the following equation:

$$\mathrm{MC}(\%) = \frac{W_D - W}{W_D} \times 100\% \quad (2)$$

where W_D is the weight of the dry beads and W is the weight of fully dried beads. Experiment was performed in triplicate for each batch and the average MC was expressed as the mean MC (%) ± standard deviation (SD).

2.7. Encapsulation and loading efficiency

Resveratrol EE and L were determined by direct method as described in our previous study (with minor modification) (Das and Ng, in press). Brief description of the method is given below.

In this method, ~25 mg beads were dissolved (pectinate network) or dispersed (resveratrol) in 5 ml of phosphate buffer (50 mM; pH 7.4) containing pectinase enzyme (2%, v/v). Methanol (10 ml) was then added to the system to ensure solubilization of poorly soluble resveratrol and mixed well on a magnetic stirrer. The mixture was centrifuged at 10,000 × g and the supernatant (contains resveratrol) was diluted to the calibration range (0.1–10 μg ml^{-1}) with methanol–water mixture (1: 1). Resveratrol content in the supernatant (or remaining in the beads) was determined by UV-Visible Spectrophotometer (UV - 1601, Shimadzu) at 320 nm. Resveratrol-free beads were used as control. EE and L of the beads for resveratrol were then determined by the Eqs. (3) and (4), respectively:

$$\mathrm{EE}(\%) = \frac{\mathrm{AQ}}{\mathrm{TQ}} \times 100\% \quad (3)$$

$$L(\%) = \frac{\mathrm{AQ}}{W_P} \times 100\% \quad (4)$$

where AQ is the actual quantity of resveratrol present in the beads, TQ is the theoretical quantity of resveratrol (initial resveratrol loading dose during the preparation of the beads), and W_P is the weight of pectin used to prepare the beads.

All experiments were performed in triplicate.

2.8. Drug release study

Because of the marginal solubility of resveratrol in SIF (Das et al., 2008), release of resveratrol in standard dissolution apparatus may not be observed even in presence of high amount of surfactant (SIF, pH 6.8 with 0.2% Tween 80 exhibited resveratrol solubility of 0.72 ± 0.13 μg ml^{-1}) (Das and Ng, in press). Further, addition of very high amount of surfactant was not at all feasible as high amount of surfactant will have a deleterious effect on the release characteristic of the beads. Hence, we devised an alternative method in our previous study (Das and Ng, in press), where we estimated the amount of resveratrol remaining in the intact beads after each time point instead of measuring the drug released in the media. This is because much of the drug is in particulate form (rather than dissolved form) due to the poor solubility of resveratrol in the release media (SIF/SGF), which will impede the proper quantification of released resveratrol. This alternative method precludes measurement of resveratrol released (either in dissolved or particulate form) in the release media.

Briefly, resveratrol-loaded beads were weighed (~25 mg) into screw cap glass test tubes and suspended in 10 ml releasing medium (preheated at 37 ± 0.2 °C). Separate tubes were employed for each time point. The tubes were kept on shaking water bath at 37 ± 0.2 °C with continuous shaking (100 rpm). Intact beads were separated from the specified tubes at selected time intervals. The beads were dissolved/dispersed in 5 ml PBS (pH 7.4, 50 mM) containing pectinase enzyme (2%, v/v) and fully dissolved by the addition of methanol (10 ml). The mixture was mixed thoroughly and centrifuged at 10,000 × g for 10 min. The supernatant (containing resveratrol) was collected, diluted with methanol–water (1: 1) to the calibration range. Resveratrol content in the supernatant (or remaining in the beads) was determined by a double beam UV spectrophotometer (UV–visible Spectrophotometer UV-1601, Shimadzu) at 320 nm. Resveratrol-free beads were used as a control.

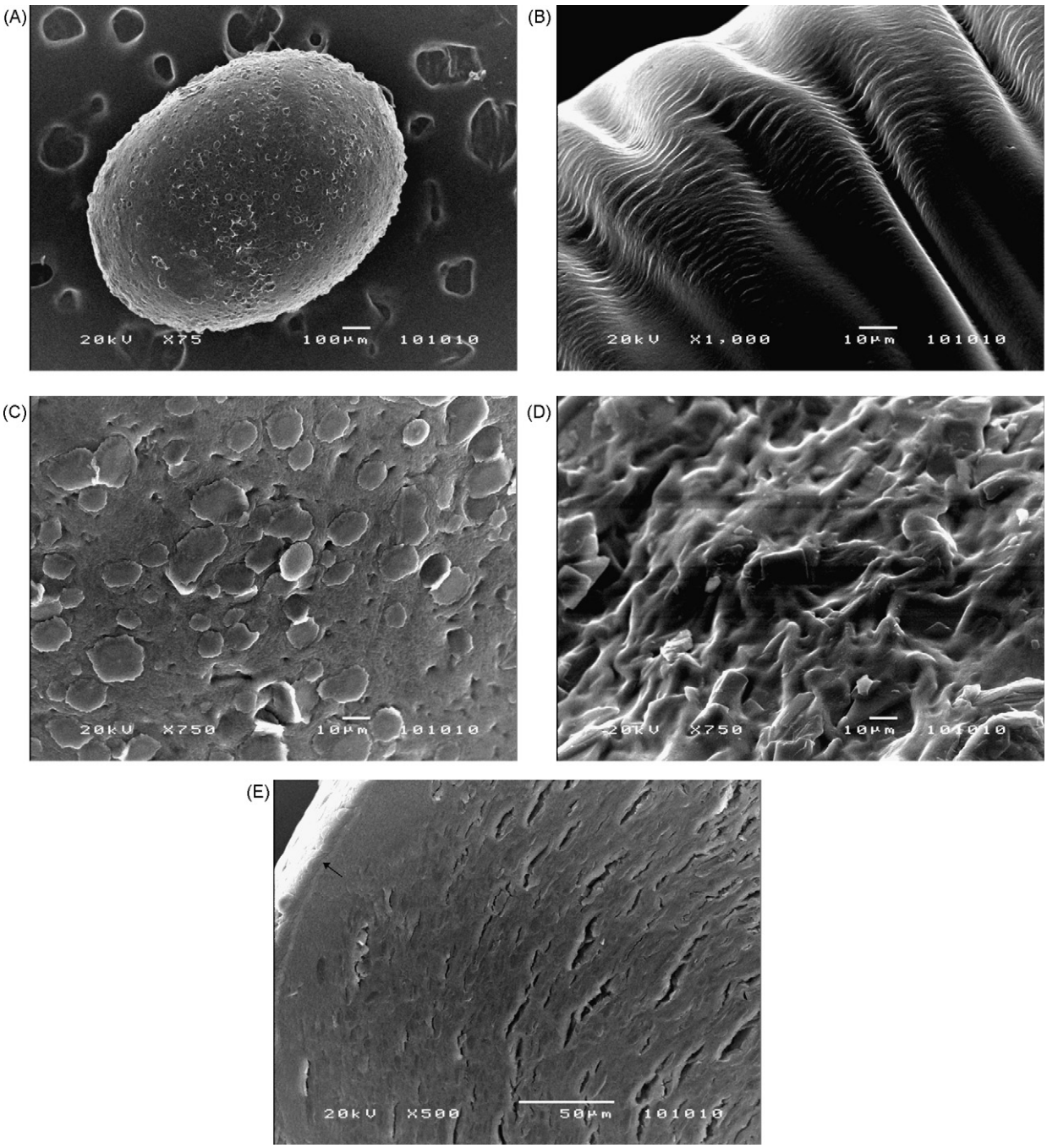

Fig. 2. Scanning electron micrographs of the surface of resveratrol-loaded bead (×75) (A), resveratrol-free bead (×1000) (B), resveratrol-loaded bead (×750) (C), resveratrol-loaded bead without PEI hardening (×750) (D), and cross-section of resveratrol-loaded bead (×500) (E). Magnifications corresponding to each figure are presented in brackets. Black arrow (E) indicates the surface layer.

To simulate the GI conditions, the following releasing media were used in subsequent manner (i.e., first medium was removed after a predetermined time point, beads were gently washed and incubated into the next medium, and so on):

2 h: Simulated gastric fluid, pH 1.2 (SGF),
2–5 h: Simulated intestinal fluid, pH 6.8 (SIF),
5–8 h: Phosphate buffer (50 mM, pH 6) with 300 PG Pectinex® Ultra SP-L (simulated colonic fluid (SCF)).

Upon oral administration, dosage forms pass through stomach (pH ~ 1.5–3.5, transit time ~ 1–2 h) and small intestine (pH ~ 5.5–6.8, transit time ~ 3–6 h) before arrival to large intestine (pH ~ 6.4–7, transit time ~ 12–24 h) (Liu et al., 2003). Therefore, the above gastric and intestinal conditions were chosen for the release study. Simultaneously, as optimum pH for the activity of pectinolytic enzyme was 3–5, a compromise pH was set for SCF (pH 6).

Percent of resveratrol remained in the intact beads (in comparison to the initial resveratrol amount in the beads) were plotted against time. All experiments were performed in triplicate.

2.9. Swelling–erosion behavior

The beads were weighed (~25 mg) and suspended in the glass test tubes containing SGF (preheated at 37 ± 0.2 °C). The test tubes

Table 2
Effect of the formulation variables on size, shape, and weight of the beads (data represents mean ± SD (n = 50)).

Variables	Values	Size (μm)		Shape (ER)		Weight of 50 beads (mg)	
		Wet beads	Dry beads	Wet beads	Dry beads	Wet beads	Dry beads
PEI concentration (%)	0	2271.23 ± 127.27	1148.68 ± 83.85	1.11 ± 0.09	1.14 ± 0.13	326.00 ± 9.41	35.95 ± 0.23
	0.05	2237.28 ± 122.18	1082.35 ± 93.45	1.06 ± 0.04	1.11 ± 0.11	326.95 ± 17.98	34.46 ± 1.96
	0.1	2233.24 ± 127.29	1047.64 ± 62.74	1.04 ± 0.01	1.06 ± 0.05	319.12 ± 5.37	33.62 ± 2.47
	0.2	2183.28 ± 119.27	1002.24 ± 82.91	1.04 ± 0.03	1.08 ± 0.07	312.73 ± 16.55	32.75 ± 3.84
	0.3	2181.25 ± 102.38	994.36 ± 72.53	1.03 ± 0.03	1.05 ± 0.05	313.19 ± 22.72	30.54 ± 1.12
Cross-linking time (h)	0.08	2218.28 ± 93.27	1058.34 ± 73.24	1.10 ± 0.09	1.14 ± 0.13	319.53 ± 3.65	34.17 ± 4.04
	0.5	2192.38 ± 106.26	1025.27 ± 83.62	1.05 ± 0.05	1.13 ± 0.09	315.24 ± 15.09	33.12 ± 3.81
	2	2183.28 ± 119.27	1002.24 ± 82.91	1.04 ± 0.03	1.08 ± 0.07	312.73 ± 16.55	32.75 ± 3.84
	24	2183.19 ± 112.16	1000.73 ± 94.38	1.03 ± 0.01	1.08 ± 0.07	317.31 ± 18.82	30.12 ± 1.65
Pectin:resveratrol	1:1	2231.27 ± 123.28	1103.73 ± 95.45	1.04 ± 0.02	1.09 ± 0.09	316.54 ± 25.62	35.31 ± 3.73
	3:1	2183.28 ± 119.27	1002.24 ± 82.91	1.04 ± 0.03	1.08 ± 0.07	312.73 ± 16.55	32.75 ± 3.84

were then placed on a shaking water bath (37 ± 0.2 °C) at 100 rpm and followed similar conditions as release study (first 2 h in SGF and subsequently in SIF for next 3 h and then in SCF). Separate test tube was assigned for each time point. The beads were separated at every time point, blotted with filter paper to remove the excess water, and weighed. The swelling–erosion ratio (SER) was calculated by the following equation:

$$\text{SER}(\%) = \frac{W_T - W_0}{W_0} \times 100\% \quad (5)$$

where W_T is the weight of the beads at the specific time point, W_0 is the initial weight of the dry beads.

Positive SER and upward trend refer to the overall swelling and weight gain of beads with water absorption, while negative SER and downward trend denote the erosion and/or drug release of the beads. Experiments were performed in triplicate. Percent SER was plotted against time to describe SER profile of the beads in the simulated GI conditions.

2.10. Stability

The beads (~25 mg) were stored for 6 months at 3 different conditions: cold (4 °C), room temperature, and accelerated temperature (40 °C). The samples were stored in triplicate for each time point. The samples are analyzed for resveratrol content at predetermined time intervals (0 day, 3 days, 7 days, 15 days, 30 days, 90 days, and 180 days) by UV spectrometer (UV–visible Spectrophotometer UV-1601, Shimadzu) using the same procedure described in the EE study.

2.11. Statistics

All statistical analyses were performed using Graph-Pad Prism Version 2.00 (San Diego, CA). All experimental data was expressed as mean ± standard deviation (SD). One-way ANOVA with the *post hoc* Tukey test or two-tail unpaired *t* test (where applicable) were performed for analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Morphology

SEM images of the beads are shown in Fig. 2A–E. Rough surface topography was observed (×75) with resveratrol-loaded beads hardened with PEI (Fig. 2A). At higher magnifications (×1000), resveratrol-free beads (Fig. 2B) showed relatively smother surface than resveratrol-loaded beads (×750, Fig. 2C). Analyses of the SEM photograph of resveratrol-loaded beads hardened with PEI revealed that the surface was covered with a rough layer due to the presence of PEI (×750, Fig. 2C), while resveratrol-loaded beads without PEI hardening did not exhibit such layer (×750, Fig. 2D). The cross-section of the resveratrol-loaded beads hardened with PEI also showed a layer (see the black arrow) at the outer surface of the beads (×500, Fig. 2E). Appearance of the beads hardened with PEI was yellowish.

3.2. Size, shape, and weight

As homogeneous dispersion of resveratrol into aqueous solution of pectin was added dropwise to the cross-linking solution, beads were formed instantly. All formulated beads were spherical in shape (ER < 1.15) and more than 2 mm in diameter, which upon drying became smaller (about 1 mm in diameter) while retained their spherical shape (Table 2). The size of the dry as well as wet beads was found to be decreased with increasing PEI concentration, cross-linking time, and pectin to resveratrol ratio. Simultaneously, with augmentation of PEI concentration, cross-linking time, and pectin to resveratrol ratio, beads (dry) became lighter (Table 2).

3.3. Weight loss during drying

Table 3 depicts the effect of the formulation variables on WL of the beads. Insignificant but little augmentation of WL was observed with increasing PEI concentration, cross-linking time, and pectin to resveratrol ratio.

3.4. Moisture content

Effect of different formulation variables on MC of the beads are listed in Table 3. The beads that were hardened with PEI exhibited significantly lower moisture content than the beads without PEI. Higher PEI concentration and longer cross-linking time produced beads with less MC. Further, higher pectin to resveratrol ratio led to production of beads with lower MC.

3.5. Encapsulation and loading efficiency

Formulation variables revealed a great influence on the EE and *L* of the formulated beads (Table 3). The data indicates the huge reductions of EE and *L* with increasing PEI concentration. The EE and *L* significantly reduced when PEI was added to the cross-linking solution in compare to the non-hardened beads. Enhancement of cross-linking time led to immense reduction of their EE and *L*. Furthermore, increase in pectin to resveratrol ratio led to formation of beads with significantly lower EE and *L*.

Table 3
Effect of the formulation variables on WL, MC, EE, and *L* of the beads (data represents mean ± SD ($n = 3$)).

Variables	Values	WL (%)[#]	MC (%)	EE (%)	*L* (%)
PEI concentration (%)	0	88.97 ± 0.25	18.90 ± 1.22[a]	98.18 ± 0.07[b]	32.73 ± 0.02[b]
	0.05	89.46 ± 0.09	12.60 ± 0.97	93.75 ± 0.72	31.25 ± 0.24
	0.1	89.46 ± 0.91	11.45 ± 0.60	79.49 ± 0.56	26.50 ± 0.19
	0.2	89.49 ± 1.57	10.43 ± 1.64	70.04 ± 0.34	23.35 ± 0.11
	0.3	90.20 ± 1.04	10.21 ± 1.27	59.53 ± 0.64	19.84 ± 0.21
Cross-linking time (h)	0.08	89.31 ± 1.22	12.32 ± 0.29	97.07 ± 1.00[c]	32.36 ± 0.33[c]
	0.5	89.45 ± 1.60	11.30 ± 1.86	80.17 ± 0.89	26.72 ± 0.30
	2	89.49 ± 1.57	10.43 ± 1.64	70.04 ± 0.34	23.35 ± 0.11
	24	90.47 ± 1.06	10.41 ± 1.02	59.50 ± 0.65	19.83 ± 0.22
Pectin:resveratrol	1:1	88.84 ± 0.95	10.74 ± 1.54	82.44 ± 1.53[d]	82.44 ± 1.53[d]
	3:1	89.49 ± 1.57	10.43 ± 1.64	70.04 ± 0.34	23.35 ± 0.11

[#] Statistical analysis is not shown.
[a] $p < 0.05$ between 0% PEI with 0.05, 0.1, 0.2, and 0.3% PEI.
[b] $p < 0.05$ among 0, 0.05, 0.1, 0.2, and 0.3% PEI.
[c] $p < 0.05$ among 0.08, 0.5, 2, and 24 h cross-linking time.
[d] $p < 0.05$ between 1:1 and 3:1 pectin to resveratrol ratio.

3.6. Drug release and swelling–erosion behavior

Effect of different formulation variables on resveratrol retention pattern and SER in the artificial GI environments are depicted in Figs. 3–5. Drug retention profile is represented by plotting resveratrol retention within the beads versus time. Simultaneously, SERs are plotted against time to indicate the swelling–erosion behaviors of the beads in simulated GI conditions. The following sections discuss the effects of different formulation parameters on resveratrol retention and swelling–erosion behaviors of the beads.

3.6.1. PEI concentration

This study revealed that PEI concentration plays a crucial role on the stability and drug retention capability of the beads during GI transit (Fig. 3A). Despite the stability of the non-hardened beads in SGF, they were rapidly degraded following their subsequent exposure to SIF (<7.5% resveratrol retained within the beads at 5 h). Beads prepared with low PEI concentration (0.05%) also demonstrated quick degradation in SIF (<39% resveratrol retained within the beads at 5 h), although they were relatively more stable than the non-hardened beads. Contrarily, high PEI concentration (0.3%) led to very strong bead formation which released a little amount of resveratrol even in SCF (>80% resveratrol retained within the beads at 8 h). Nevertheless, beads formulated with 0.2% PEI gave rise to beads that were stable in SGF and SIF (>90% resveratrol retained within the beads at 5 h), and slowly released the drug in SCF (>58% resveratrol retained within the beads at 8 h). On the other hand, formulation with 0.1% PEI produced beads that were moderately

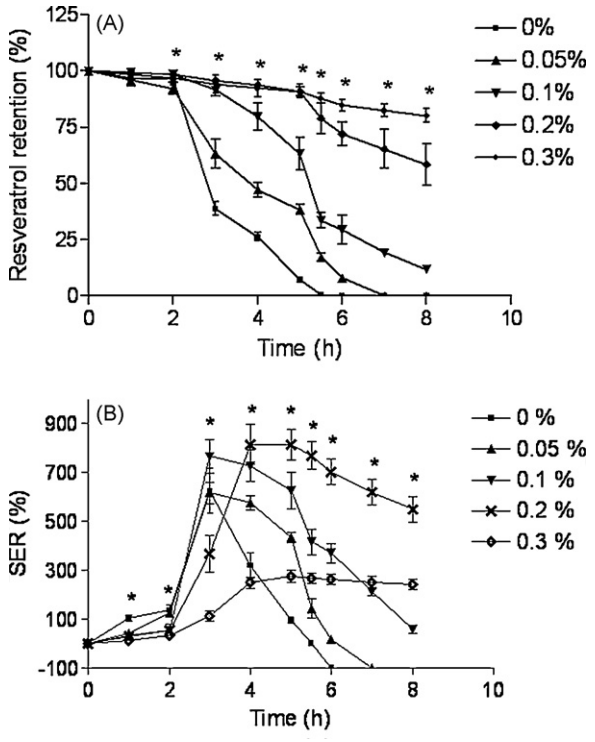

Fig. 3. Effect of PEI concentration on retention of resveratrol within the beads (A) and SER of the beads (B). The beads were incubated in simulated GI conditions (2 h in SGF, 3–5 h in SIF, 6–8 h in SCF). SER value of −100 represents complete dissolution of the beads. Data presented as mean ± SD. $^{*}p < 0.05$.

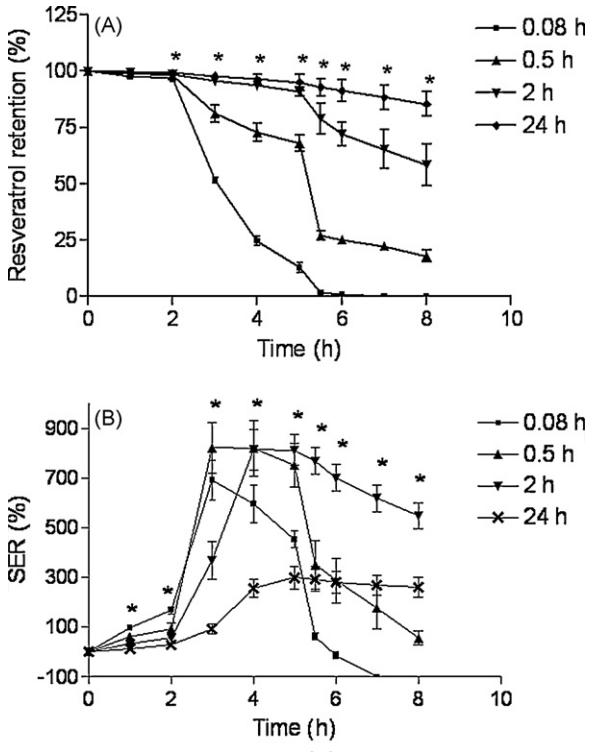

Fig. 4. Effect of cross-linking time on retention of resveratrol within the beads (A) and SER of the beads (B). The beads were incubated in simulated GI conditions (2 h in SGF, 3–5 h in SIF, 6–8 h in SCF). SER value of −100 represents complete dissolution of the beads. Data presented as mean ± SD. $^{*}p < 0.05$.

stable in SIF (>63% resveratrol retained within the beads at 5 h) but very quickly degraded in SCF (~12% resveratrol retained within the beads at 8 h).

Huge swelling was observed for the beads prepared with 0.2% PEI, whereas beads prepared with 0.3% PEI exhibited very low swelling (Fig. 3B). On the other hand, beads prepared with 0–0.1% PEI revealed an early phase of swelling followed by erosion. However, SER increased with PEI concentration 0–0.2%.

3.6.2. Cross-linking time

Similar to PEI concentration, cross-linking time also had decisive influence on beads' stability in simulated GI fluids (Fig. 4A). A very short cross-linking time (0.08 h) produced beads that were degraded rapidly in SIF (<13% resveratrol retained within the beads at 5 h), whereas a long cross-linking time (24 h) led to production of very strong (stable) beads which remained almost intact even in SCF (>85% resveratrol retained within the beads at 8 h). In contrast, intermediate cross-linking time (0.5 and 2 h), produced beads that showed moderate stability in SIF (~68% resveratrol retained within the beads at 5 h) but rapidly degraded in SCF (~18% resveratrol retained within the beads at 8 h), and beads that were stable in SIF (>90% resveratrol retained within the beads at 5 h) but degraded slowly in SCF (>58% resveratrol retained within the beads at 8 h), respectively. However, all formulated beads were stable in SGF (>96% resveratrol retained within the beads at 2 h).

SER was very low for beads cross-linked for 24 h, while an early phase of swelling followed by rapid erosion was observed in case of beads cross-linked for 0.08 h (Fig. 4B). Simultaneously, intermediate cross-linking time (0.5 and 2 h) produced beads with high SER. In general, SER decreased with increasing cross-linking time (0.5–24 h).

3.6.3. Pectin to resveratrol ratio

The data indicates that both tested pectin to resveratrol ratio had no influence on resveratrol retention within the beads (Fig. 5A) and swelling–erosion behavior (Fig. 5B). Both formulations established their stability in SIF (>87% resveratrol retained within the beads at 5 h) and slow degradation in SCF (>52% resveratrol retained within the beads at 8 h). This study demonstrates that a very high percentage of resveratrol can be encapsulated in Ca-pectinate beads without affecting their drug retention and SER properties.

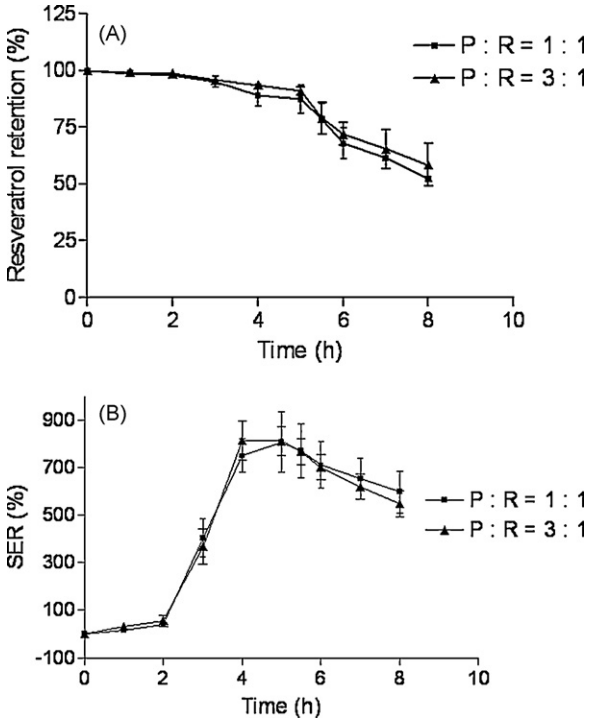

Fig. 5. Effect of pectin to resveratrol ratio (P:R) on retention of resveratrol within the beads (A) and SER of the beads (B). The beads were incubated in simulated GI conditions (2 h in SGF, 3–5 h in SIF, 6–8 h in SCF). Data presented as mean ± SD. $^{*}p < 0.05$.

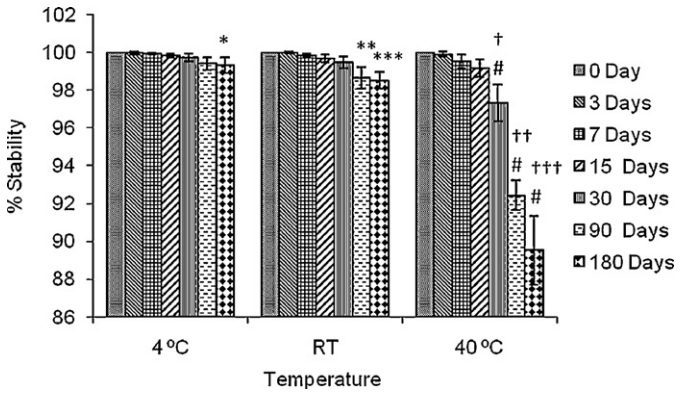

Fig. 6. Stability of resveratrol in the optimized resveratrol-loaded Ca-pectinate beads. Data represents mean ± SD. $^{*}p < 0.05$ for the difference between 180 days with 0, 3, and 7 days, $^{**}p < 0.05$ for the difference between 90 days with 0, 3, 7, and 15 days, $^{***}p < 0.05$ for the difference between 180 days with 0, 3, 7, 15, and 30 days, $^{\dagger}p < 0.05$ for the difference between 30 days with 0 and 3 days, $^{\dagger\dagger}p < 0.05$ for the difference between 90 days with 0, 3, 7, 15, and 30 days, $^{\dagger\dagger\dagger}p < 0.05$ for the difference between 180 days with 0, 3, 7, 15, 30, and 90 days, $^{\#}p < 0.05$ for the difference between 40 °C with 4 °C and RT.

3.7. Stability

The stability profile of the formulated beads at different storage conditions is presented in Fig. 6. The formulated beads were found to be stable for 6 months at 4 °C (>99%) and RT (>98.5%), although stability was low at 40 °C (>90%).

4. Discussion

Pectins with DE < 50% (i.e., LM pectins) contain more free carboxylic group than high methoxy pectins (HM pectins). Therefore, more cross-linking between divalent cations (e.g., Ca^{2+}) and free carboxylic groups of the pectins is evident in LM pectins than HM pectins (Liu et al., 2003). Moreover, because of the reduced hydrophilicity of pectins in presence of amide groups, amidated pectins are more prone to react with calcium ion than non-amidated pectins. Additionally, hydrogen bonding between amide groups also increases gel strength in amidated pectins (Thakur et al., 1997). These interactions in amidated LM pectins lead to the formation of a more compact Ca-pectinate network than non-amidated and/or HM pectins. Hence, amidated LM pectin was chosen for the formulation development purpose. In addition, together with their more reproducible and predictable GI transit time, more reliable drug release profile, and less local irritation than single unit dosage forms (Maestrelli et al., 2008a), multiple unit dosage forms of enzyme specificity quickly spread out upon their arrival to the colon. This phenomenon leads to rapid drug release from the multiple unit dosage forms at the colon due to an enhanced surface area being exposed to the enzymes (Rodriguez et al., 1998). Thus, multi-particulate Ca-pectinate beads were selected in the present study.

In the current study, when the highly water insoluble resveratrol was homogeneously dispersed into the aqueous solution of pectin and subsequently added dropwise to the counter ion solution of calcium, ionic interaction between the negatively charged carboxylic groups on pectin molecules and the positively charged divalent

calcium ions led to intermolecular cross-linking and instantaneously produced gelled sphere. Moreover, presence of PEI in the cross-linking solution further induced cross-linking between pectin chains and PEI, which helped in the formation of stronger beads (this will be discussed later in detail).

The spherical beads were easily prepared without any sophisticated instrument. The morphological examination exhibits rougher surface of resveratrol-loaded beads than resveratrol-free beads. This might be due to presence of resveratrol crystals that are embedded in the polymer matrix of resveratrol-loaded beads. A clear difference between the surface of the beads hardened with PEI and non-hardened beads was observed. This indicates formation of an additional layer on the surface of the beads hardened with PEI. SEM image of the cross-section also reveals a thin layer on the bead surface, which is anticipated due to surface hardening by PEI. Because of the high molecular weight of PEI, it was probably unable to penetrate inside the Ca-pectinate network and was therefore localized on the bead surface.

As the diameter (25 G) of the needle used to prepare the beads and drying method (RT) were constant throughout the experiment, it is obvious that other formulation variables were affecting the particle size of the dried beads. This might be due to more compact beads were produced (probably because of pronounced gel bead shrinkage caused by syneresis) by increasing PEI concentration in cross-linking solution and increasing cross-linking time, which led to smaller and lighter beads with higher WL and lower MC. Moreover, beads with lower pectin to resveratrol ratio led to bigger and heavier particles with higher MC and lower WL. This is anticipated as lesser amount of pectin was available in theses beads because of lower polymer to drug ratio.

Generally, EE and *L* of the beads depend on the dissolution of resveratrol into the cross-linking solution. Resveratrol dissolution in the cross-linking solution increases with increased PEI concentration and residence time, which ultimately led to decreased EE and *L*. Moreover, compact bead formation and pronounced gel bead shrinkage (syneresis) at the higher PEI concentration and longer cross-linking time probably expelled some resveratrol from the beads, leading to lower EE and *L*. In general, decrease of polymer to drug ratio leads to lower EE and *L* (Sriamornsak and Nunthanid, 1998). In contrary, EE and *L* increased with decreasing pectin to resveratrol ratio. This might be due to the following reasons. After certain amount, resveratrol reaches saturation in the solution at constant temperature. Therefore, when high amount of resveratrol was added into the pectin solution, only limited portion (amount required to saturate the cross-linking solution) of it came out from the beads to the cross-linking solution, which consequently led to higher EE and *L*.

Pre-exposure to acidic medium drastically affects the beads' behavior and decreases its resistance in SIF (basically a phosphate buffer, pH 6.8). In the gastric fluid, carboxylic groups of pectin chains at the beads' surface convert to COOH, as the pH of the medium (1.5) is lower than pK_a of pectin (3.5) (Ahmed, 2005). Simultaneously, displacement of calcium ion (Ca^{2+}) with H^+ or Na^+ occurs at the outer layers of the beads. Thus cross-linked Ca-pectinate network is depleted of its cross-linking agent. But, due to less solubility in acidic fluid and lack of repulsion between carboxylic groups, beads retain their relatively insoluble and tightly packed spherical structure. Thus, less swelling–erosion and drug release take place in SGF. However, the COOH groups reconvert to COO^- upon subsequent exposure to SIF (pH 6.8), which repel each other. As some Ca^{2+} already depleted from the Ca-pectinate network, a drastic swelling–erosion and subsequent drug release occurs (Atyabi et al., 2005). In addition, drug release from Ca-pectinate beads in SIF is further accelerated due to solvent penetration into the Ca-pectinate network, followed by ion exchange between Ca^{2+} and Na^+/K^+. These lead to extended and swelled bead structure and partially forming soluble pectin regions, which are more permeable (Sriamornsak, 1998). The whole process is called as 'acid–base attack'. These observations compelled us to modify the beads to obtain the desired profiles.

The increased resveratrol retention in upper GI conditions in case of beads prepared with increasing concentration of PEI was probably due to the promotion of cross-linking between pectin chain and PEI. A dense surface was formed in presence of more than 0.1% PEI. Similarly, longer cross-linking time provides more time for cross-linking of the Ca-pectinate network and hardening of the bead surface, which led to formation of stronger beads. The PEI cross-linking made the Ca-pectinate network more stable and prevented the easy dissolution of Ca-pectinate network in the higher pH of the intestinal fluid. In general, cationic polymers have tendency to react with pectin chains and form polyelectrolyte complex. As PEI is a cationic polymer, it cross-links with the free carboxylic groups of the Ca-pectinate network. The basic mechanism of this cross-linking is based on the covalent interaction between negatively charged carboxylic groups of pectin chains and positively charged amine groups of PEI. Due to the high density of the Ca-pectinate network and high molecular weight of PEI (molecular weight ~25,000), PEI could not penetrate into the core matrix and remained localized on the bead surface. This phenomenon led to produce a relatively hard shell on the bead surface. The swelling and drug release of these beads were in a controlled manner unlike the beads prepared without PEI. Low PEI concentration and cross-linking time led to the formation of weakly cross-linked beads, which absorbed more water and swelled.

Furthermore, 0.2% PEI in cross-linking solution and cross-linking time of 2 h was sufficient to produce strong beads which were able to load very high amount of resveratrol (pectin to resveratrol ratio = 1:1) and revealed similar resveratrol retention pattern (in simulated GI conditions) to the beads prepared with lower amount of resveratrol (pectin to resveratrol ratio = 3:1).

However, the release study exhibited that pectinolytic enzyme was able to degrade the beads despite PEI cross-linking, although degradation was slow in case of the beads prepared at high PEI concentration or long cross-linking time. Pectinase enzyme most likely attacks the pectin chains of the Ca-pectinate–PEI complex on bead's surface and degrades the protective upper layer. Then the enzyme gets access to the core of the beads and gradually degrades the bead structure, which leads to the release of encapsulated drug. We anticipate that resveratrol released from the core matrix as undissolved particles due to its negligible solubility in the release media (Das and Ng, in press). As the resveratrol particles fall off from the beads, pores are created in the matrix that allow more fluid penetration in to the matrix and most likely more resveratrol particles fall off and the bead matrix erodes. Formation of strong matrix and hard surface layer slow down the release of encapsulated drug. Consequently, stability data indicates that beads are stable when stored at 4 °C and RT.

To our knowledge, one research group (Bourgeois et al., 2005, 2008) had earlier exploited the role of PEI on β-lactamases loaded pectin bead formation. But, their formulation and evaluation procedures were totally different form our study. They prepared the drug-loaded Ca-pectinate beads first and then hardened the preformed beads in PEI solution. Most importantly, they did not perform release study in actual simulated GI conditions. That means they missed out the acid–base attack phenomenon which will be obvious in *in vivo* condition. In our current study, when PEI was added in the cross-linking solution, cross-linking of pectin chains with Ca^{2+} and hardening of the bead surface occurred simultaneously with bead formation. This led to elimination of surface hardening of the preformed beads (hence, hardening time), which shortened the production time. Thus, our formulation procedure is unique in the sense that it reduced production cost by cutting

down extra steps of washing and hardening, and by decreasing production time and manpower requirement. Moreover, sufficiently strong resveratrol-loaded beads were produced by this procedure, which could resist acid–base attack in the GI tract, which is required for colon-specific delivery. The researchers (Bourgeois et al., 2005, 2008) also used very high amount of PEI to prepare their formations (0.6–1%). Our data indicates that only a small amount of PEI (0.1–0.2%) is needed for the formulation of resveratrol-loaded colon-specific Ca-pectinate beads. Since low concentration of PEI was used during bead preparation and the excess amount was washed off after cross-linking, very less amount of PEI is likely to remain in the beads. In addition, our formulations can encapsulate very high amount of drug (pectin to resveratrol ratio = 1:1).

5. Conclusion

Because of its rapid absorption and metabolism at the upper GI tract, development of a colon-specific delivery system of resveratrol becomes indispensable for the treatment of colonic diseases such as colorectal cancer, colonic inflammation etc. (where it is potentially active). Hence, resveratrol-loaded Ca-pectinate beads were developed as multi-particulate colon-specific delivery system. Beads prepared with 0.1% PEI and hardened for 2 h or with 0.2% PEI and hardened for 0.5 h were observed to be the best formulations as they could encapsulate more than 80% of the drug and at the same time showed desirable drug release pattern. However, beads prepared at 0.2% PEI concentration and cross-linked for 2 h also demonstrated a desirable colon-specific drug release pattern, although EE was little low. Very high amount of resveratrol can be encapsulated into these beads without altering the resveratrol retention pattern in simulated GI conditions. Because PEI was added in the cross-linking solution, hardening of the bead surface occurred simultaneously with bead formation, which eliminated extra formulation steps, and reduced the production time and cost. Moreover, a small amount of PEI is required to prepare the beads with desired drug release profile. Though our study showed that the beads were able to prevent release of resveratrol in simulated upper GI conditions and release at simulated colonic condition, further *in vivo* investigation is essential to prove this *in vitro* prediction.

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