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Bioadhesive chitosan nanoparticles: Preparation and characterization

Anitha R. Dudhani[a,b,c,*], Shantha L. Kosaraju[a]

[a] CSIRO Food and Nutritional Sciences, 671 Sneydes Road, Werribee, Victoria 3030, Australia
[b] Department of Pharmacy Practice, Monash Institute of Pharmaceutical Sciences Monash University, 381 Royal Parade, Victoria - 3052, Australia
[c] School of Biomedical and Health Science, Victoria University, Building 6, Mckechnie Street, ST Albans, Victoria - 3021, Australia

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ABSTRACT

Bioadhesive chitosan nanoparticles (CS NPs) were prepared for encapsulation of catechin and evaluation of their mucoadhesive potential that leads to enhanced oral bioavailability of catechin. CS NPs and catechin loaded CS NPs were obtained by ionic gelation between the CS and sodium tripolyphosphate (TPP). Particle size distribution analysis confirmed the size ranges, 110 ± 5 nm and 130 ± 5 nm for CS NPs and catechin loaded CS NPs, respectively. TEM indicated smooth and spherical nanoparticles. FTIR and DSC showed no significant interactions between catechin and CS after encapsulation and cross-linking. Entrapment efficiency of 90% was achieved with a weight ratio of 2:1 (CS:TPP) at pH 5.5. *In vitro* release of catechin from CS NPs was 32% within 24 h and exhibited 40% and 32% mucoadhesivity for catechin loaded CS NPs and CS NPs, respectively, demonstrating potential for controlled release of catechin in GIT.

1. Introduction

Concept of polymeric drug delivery system, initially established by Ringsdorf, has potential for delivering the drug to a definite target (Andrianov & Payne, 1998). Generally, oral route is the most preferred drug delivery route but, it has certain limitations especially for hydrophilic drugs due to [a] low bioavailability (premature degradation, metabolism and/or poor solubility of drugs in gastrointestinal tract (GIT)), [b] inadequate intestinal transit time to facilitate transcytosis and [c] dependence on paracellular transport (Kotze et al., 1998). The tight junctions located in epithelial cells limit the transport of hydrophilic molecules in paracellular transport (Borchard et al., 1996).

Nanoparticles (NPs) are well known to transport and deliver drugs which are unstable in the biological fluids and cannot readily diffuse across the mucosal barrier (Alonso, 2004; Prego et al., 2005). Oral NPs are promising drug delivery systems due to improved bioavailability, targetability, bioadhesion and controlled release of drugs in GIT (Chen & Langer, 1998; Kawashima, 2001; Nefzger, Kreuter, Voges, Liehl, & Czok, 1984; Pinto, Neufeld, Ribeiro, & Veiga, 2006). They can transit directly and/or adhere to the mucosa, which is a prerequisite step before the translocation process of particles. Hence, bioadhesion plays a key role to deliver drugs across the epithelia, avoiding hepatic first pass metabolism and enzymatic degradation in the GIT.

Chitosan (CS) is a naturally occurring nontoxic, biocompatible, biodegradable, cationic polysaccharide (Onishi & Machida, 1999; Rao & Sharma, 1997). This hydrophilic polymer can easily cross-link with counter poly anions like TPP to control the release of drugs (Hu et al., 2002). CS is a mucoadhesive polymer (Janes, Calvo, & Alonso, 2001; Lehr, Bouwstra, Schacht, & Junginger, 1992) with permeation enhancing properties (Porporatto, Bianco, & Correa, 2005) which facilitate opening of the epithelial tight junctions (Artursson, Lindmark, Davis, & Illum, 1994; Luessen et al., 1996; van der Lubben, Verhoef, Borchard, & Junginger, 2001; van der Lubben, Verhoef, van Aelst, Borchard, & Junginger, 2001; Yamamoto, Kuno, Sugimoto, Takeuchi, & Kawashima, 2005).

Flavonoids like catechin are polyphenolic compounds (PCs) with antioxidant properties, which efficiently chelate trace metals and prevent lipid peroxidation (Gilgun-Sherki, Melamed, & Offen, 2001). PCs are hydrophilic compounds which can undergo extensive first pass metabolism (Manach & Donovan, 2004) in GIT and the biological activities of metabolites are not fully understood (Paul, Joanna, Russell, & David, 2004; Spencer, Abd El Mohsen, & Rice-Evans, 2004). Natural antioxidants including PCs and tocopherols, have been shown to undergo loss of activity at lower concentration and to become pro-oxidants at high concentrations (Frankel, Huang, Kanner, & German, 2002). Hence, controlled or sustained delivery is crucial to obtain maximum benefits of using PCs as nutraceuticals. Although, previous studies were carried out on catechin encapsulated CS particulates, detailed investigation on *in vitro* release behavior and bioad-

* Corresponding author at: School of Biomedical and Health Science, Victoria University, Building 6, Mckechnie Street, St. Albans, Victoria - 3021, Australia.
Tel.: +61 3 9919 2711; fax: +61 3 9919 2465.
E-mail address: anitha.dudhani@live.vu.edu.au (A.R. Dudhani).

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hesive properties have not been reported (Zhang & Kosaraju, 2007).

This study reports the preparation, evaluation and characterization of CS NPs with or without catechin and their controlled release properties. Fluorescein isothiocyanate (FITC) labeled CS were synthesized and NPs were prepared to evaluate the mucoadhesion properties.

2. Materials and methods

2.1. Materials

Low molecular weight (LMW) CS (20–200 cPs, 75–85% degree of deacetylation), (+)-catechin hydrate (minimum 98% by TLC), lyophilized chitosanase powder (>50 units/mg protein from *Streptomyces griseus* strain, 204.95 units/mg), lysozyme (from chicken egg white, 78,643 U/mg, Fluka Biochemica) and fluorescein isothiocyanate (FITC) ~90% HPLC grade, were obtained from Sigma–Aldrich, Australia. Sodium tripolyphosphate was obtained from Ajax Chemicals, Australia.

2.2. Preparation of CS NPs

CS NPs were prepared by Calvo method (Calvo, Remuñán-López, Vila-Jato, & Alonso, 1997). CS solutions (0.05% or 0.1% (w/w)) were prepared with glacial acetic acid, 1.75 times that of CS weight. TPP (0.1% (w/w)) was added (weight ratios of CS:TPP at 2.5:1 and 2:1) drop wise to CS solution with magnetic stirring at 750 rpm and continued stirring for 60 min. The resultant suspension was subjected to particle size analysis. Further, CS NPs were prepared (modified Calvo method) by altering pH of TPP to obtain optimal particle size. Sample composition of 0.1% (w/w) of CS and 0.1% (w/w) of TPP at weight ratio of 2:1 and pH 5.5 were selected for further studies. For comparative analysis, CS NPs with TPP of pH 4.5 were prepared. Particles formed were washed and ultracentrifuged using Beckmann-Coulter® optima-L-90K ultracentrifuge USA, at 74,088 × *g* for 80 min at RT. The pellets were immediately freeze dried at −40 °C (10^{-2} Torr) for 48 h using Dynavac® FD-5 freeze drier, Australia. Both CS NPs and supernatant were stored at 4 °C until further analysis.

2.3. Preparation of catechin loaded CS NPs

Catechin loaded CS NPs were prepared by incorporating catechin (50% (w/w of CS)) in 0.1% (w/w) CS by magnetic stirring at 50–60 °C for 30 min followed by similar process explained in Section 2.2. Three independent batches of CS NPs with or without catechin were prepared for physiochemical characterization and results presented as mean ± SD.

2.4. Flocculation studies

NPs after preparation were subjected to flocculation studies by allowing them to stand (without stirring) over 24 h at RT and sampled at 0, 4, 8 and 24 h intervals for particle size analysis.

2.5. Particle size, charge and morphology of NPs

Measurement of particle size, zeta potential and polydispersity (size distribution) of NPs were performed using Zetasizer® Nano ZS 90 (Malvern Instruments, UK) by dynamic light scattering technique (using Smoluchowski equation, at a detector angle of 90°, wavelength of 633 nm at 25 °C). Measurements were carried out in dilute acetic acid medium. The morphological examination and particle size of the freeze dried NPs were determined by TEM (Jeol® 100S Japan, 80 KV). The lyophilized particles (100 μg) were diluted with deionised water (2 ml) and sonicated for 2 min. Samples for TEM imaging were prepared by placing a drop of colloid dispersion containing CS NPs (with or without catechin) on the formvar coated copper grid and air dried.

2.6. Fourier transform infrared (FTIR) and differential scanning calorimetry (DSC)

FTIR analyses for CS NPs (with or without catechin) were performed using a FTIR spectrometer (FTIR-8400S, Shimadzu®, Japan). Freeze dried sample (7.0–9.0 mg) was placed on IR crystal window and subjected to light within the infrared region. The instrument was operated with resolution of 4 cm^{-1} and 128 scans with frequency range of 400–4000 cm^{-1}.

DSC analysis of pure CS, freeze dried CS NPs, freeze dried catechin loaded CS NPs, physical mixture (CS + catechin) and pure catechin were carried out using PerkinElmer® DSC 7, USA, calibrated with indium. Sample (5 mg) was placed onto a standard aluminum pan, crimped and heated from 60 to 400 °C at a constant rate of 10 °C per min under continuous purging of nitrogen (20 ml/min). An empty sealed pan was used as reference. All samples were run in triplicate and the mean average values were calculated.

2.7. Determination of entrapment efficiency, loading capacity and percentage yield

The drug content in NPs was calculated from the difference between the total amount of drug added in the NP preparation and the amount of unentrapped drug in the aqueous medium. The catechin content was analyzed by modified Folin-Ciocalteu (FC) method. In brief, 200 μl of samples were mixed with 140 μl of 0.2N FC reagent, 2.4 ml of deionised water and 420 μl of sodium carbonate (20%). The mixture was placed in dark at ambient condition for 1 h, to which 910 μl of deionised water was added and absorbance was measured using UV–vis spectrophotometer (Pharma Spec uv-1700, Shimadzu® Japan) at 765 nm (Roura, Andres-Lacueva, Estruch, & Lamuela-Raventos, 2006).

Encapsulation efficiency (EE) was calculated using equation:

$$\text{EE}\ (\%) = \frac{\text{TC} - \text{FC}}{\text{TC}} \times 100$$

where TC is the total amount of catechin and FC is the free amount of catechin in the supernatant.

Loading capacity (LC) was articulated as:

$$\text{LC}\ (\%) = \frac{\text{TC} - \text{FC}}{\text{wt of the NPs retrieved}} \times 100$$

Percentage yield (w/w) was calculated from the weight of dried NPs recovered (W_1) and the sum of the initial dry weight of starting materials (W_2) as:

$$\text{Yield}\,(\%) = \frac{W_1}{W_2} \times 100$$

Entrapment efficiency was also determined by enzymatic breakdown of CS NPs loaded with catechin using combination of chitosanase and lysozyme. Chitosanase (0.08–0.10 g/l) and lysozyme (0.01 g/l) were used to digest 5 mg of catechin loaded CS NPs in 30 ml of sodium acetate buffer (50 mM, pH 5.5) and incubated at 37 °C for 60 h (Mao et al., 2001). An aliquot of 2 ml were drawn, with syringe (attached with 0.22 μm membrane filter), at predetermined time intervals 0, 6, 24, 32 and 60 h, and analyzed for catechin using FC reagent method as mentioned above in this section. An equivalent amount of the fresh medium (pre-warmed to 37 °C) was replaced each time after withdrawing sample for analysis.

2.8. Swelling studies

Water sorption capacity of CS NPs (with or without catechin) was determined by swelling the NPs in media of pH 1.2 (HCl; potassium chloride), 4 (acetate buffer) and 6.8 (phosphate buffer) at RT for 24 h. The pre-weighed NPs (50 mg) were placed in each media and analyzed at 0 h, 4 h, 8 h, 12 h or 24 h intervals. Samples were centrifuged (74,088 × *g* at RT) for 2 h in pre-weighed centrifuge tubes and the wet weights were determined after decanting the supernatant. The percentage swelling of NPs was then calculated gravimetrically using the following formula:

$$E_{sw}\ (\%) = \frac{W_w - W_o}{W_o} \times 100$$

where E_{sw} is the percent swelling of NPs, W_o is the initial weight of NPs and W_w is the wet weight.

2.9. In vitro release studies

Catechin loaded CS NPs (25 mg) were placed into previously soaked cellulose dialysis bag (MWCO 12–14 kDa, Spectra/Por®4, Spectrum Labs, CA, USA) containing 3 ml of enzyme free simulated gastric fluid. The release of an equivalent amount of control samples (pure catechin and CS NPs) was carried out in the dialysis bags for the same time points. The dialysis bag was placed in beaker containing 60 ml of enzyme free simulated fluids (prepared according to the US Pharmacopoeia) and incubated in a thermostatically controlled (37 °C) shaking water bath at 100 rpm. An aliquot of 5 ml were drawn with syringe (attached with 0.22 μm membrane filter), at predetermined time intervals 0, 1 and 2 h from simulated gastric fluid (SGF) followed by 4 h, 8 h, 12 h, 24 h and 48 h from enzyme free simulated intestinal fluid (SIF). An equivalent amount of fresh simulated medium (pre-warmed at 37 °C) was replaced each time after withdrawing sample for analysis of catechin by FC method as mentioned in Section 2.7.

2.10. Mucoadhesion studies

FITC CS was prepared based on the method followed by Lin et al. to evaluate the mucoadhesive property of CS NPs with or without catechin (Lin et al., 2005). To determine the labeling efficiency, fluorescence intensity of a solution of FITC CS dissolved in 0.1 M acetic acid was diluted with phosphate buffer (pH 8), to yield a final concentration of 5 μg/ml. Labeling efficiency (%) was calculated as the amount of FITC measured to the amount of FITC CS. The calibration range for FITC was 0.002–0.008 mg/l prepared by diluting 100 μg/ml methanolic solution of FITC with phosphate buffer (pH 8) (Min, Eugene, & Lee-Yong, 2004). FITC labeled CS NPs with or without catechin were prepared by similar method discussed for CS NPS and catechin loaded CS NPs as explained in Section 2.2 and analyzed for mean particle size and zeta potential.

Freshly excised porcine intestinal tissues of 4 cm length were obtained from local abattoir. The tissues were washed internally and externally with isotonic saline. One end of the inverted intestinal tissue was tied to make a pouch which was filled with intestinal fluid. The other end was tied and entire tissue was immersed in a tube containing known weight of FITC labeled CS NPs (30 mg), in 10 ml of SIF of pH 6.8. The tube was agitated end over end in a thermostatically controlled shaking bath (37 °C) for 30 min at 125 rpm. The tissues were removed from the tube and immersed in 10 ml of lysozyme solution (10 μg/ml) dissolved in acetate buffer, pH 5.5 and incubated in a water bath (35 °C) for 24 h to breakdown the particles. The solutions were centrifuged at 74,008 × *g* for 80 min and supernatant was analyzed for fluorescence by spectrofluorometer (RF-5301PC, Shimadzu®, Japan) at λ_{exc} 490 nm and λ_{emi} 520 nm. All recordings were averaged from five determinations. The calibration curve of FITC CS NPs was carried out by incubating with tissues in acetate buffer of pH 5.5.

Percentage mucoadhesion (MA) was calculated as below:

$$\%\,MA = I - F \times 100$$

where '*I*' is the initial fluorescence and *F* is the fluorescence after treatment.

Table 1
Average particle size of CS NPs obtained with varying concentrations of CS and TPP/weight ratios without pH adjustment of TPP (pH 9.2).

Concentration of CS/TPP	Weight ratio	PDI ($n = 6$)	Particle size (nm)
0.05% w/w CS 0.05% w/w TPP	2:1	0.46	114 ± 08
0.05% w/w CS 0.1% w/w TPP	2.5:1	0.44	152 ± 10
0.1% w/w CS 0.1% w/w TPP	2:1	0.43	176 ± 12
0.1% w/w CS 0.1% w/w TPP	2.5:1	0.57	192 ± 13

3. Results and discussion

3.1. Preparation of NPs

Two concentrations of CS (0.05% or 0.1% (w/w)) with weight ratios of 2:1 and 2.5:1 (CS:TPP) resulted in formation of NPs sized below 200 nm (poly dispersity index <1) as shown in Table 1. Sufficient charge density is necessary for anions to cross-link CS and form particles. Decrease in pH of TPP to acidic range, decreases the charge density (Shu & Zhu, 2002). NP formation was observed previously only at specific concentrations of CS (1–3 mg/ml) and TPP (1 mg/ml) (Wu, Yang, Wang, Hu, & Fu, 2005; Xu & Du, 2003). It is evident from our findings, particle size range of 110 ± 5 nm (CS NPs) and 130 ± 5 nm (catechin loaded NPs) can be produced with a critical concentration of 0.1% CS and TPP at 2:1 weight ratio and pH 5.5 (Figs. 1B, 2A and 2B). Particle size 168 ± 5 nm was obtained with concentration of 0.1% CS and TPP at 2:1 weight ratio and pH 4.5 (Fig. 1A). Our results furthermore indicated an increase in particle size with increase in TPP concentration and weight ratios, which is concurrent to the findings of Calvo et al. (Table 1). CS in acidic media (pK_a 6.5) can interact with the negatively charged TPP, forming inter- and intra-molecular cross-linkages, yielding ionically cross-linked CS NPs (De Campos, Sanchez, & Alonso, 2001; Dumitriu & Chornet, 1998; Mi, Shyu, Lee, & Wong, 1999; Xu & Du, 2003). This method results in spontaneous formation of NPs of smaller size with positive charge (De Campos et al., 2001; Pan et al., 2002) without using any organic solvent or surfactants at RT (Hu et al., 2002). Manipulation of particle charge by variation of pH (Zhang, Ping, Ding, Cheng, & Shen, 2004) can also be achieved. The potential drawback is that, under low and high pH conditions, there may be disintegration of the particles due to weak interaction between CS and TPP (Zhang & Kosaraju, 2007).

Aggregation studies of CS NPs were conducted over a period of 24 h. CS NPs are thermodynamically unstable at certain unfavorable pH conditions and at high particle concentrations because of high surface energy associated with the nanosize range. Major growth of particle size after ionic gelation was reported to be complete within 60 min followed by a slight increment over 24 h (Gan, Wang, Cochrane, & McCarron, 2005). LMW CS (0.1% (w/w)) with TPP solution (0.1% (w/w)) at 2:1 weight ratio produced a stable nanoparticulate system with minimal size increase by 6 nm and PDI by 0.061, over 24 h. High molecular weight CS produced larger particles or aggregates (Zhang & Kosaraju, 2007).

TPP dissolved in water dissociates to hydroxide (OH^-) and tripolyphosphoric ($P_3O_{10}^{5-}$ and $HP_3O_{10}^{4-}$) ions (pH 9). At basic pH, both hydroxide and tripolyphosphoric ions could competitively react ionically with the protonated amino group (NH^{3+}) of CS solu-

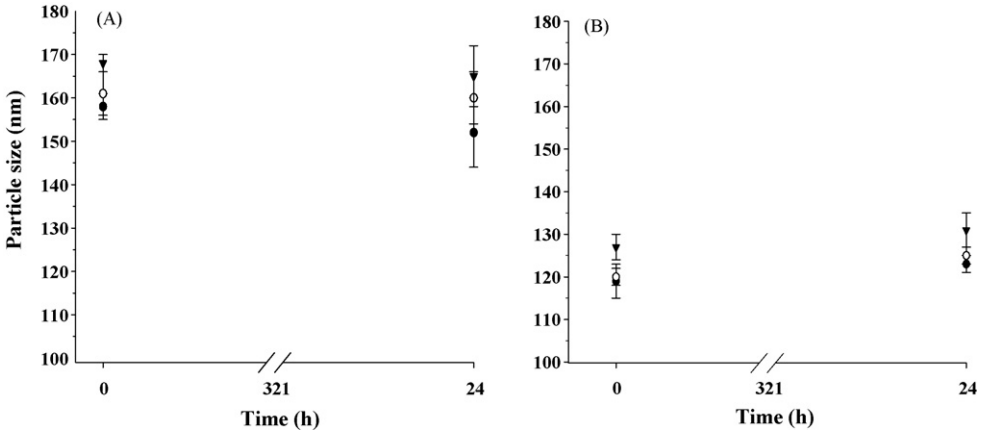

Fig. 1. Aggregation studies of CS NPs at (A) pH 4.5 (B) pH 5.5.

tion (pH 3.5) by deprotonation or ionic cross-linking. By adjusting pH of TPP to acidic pH i.e., below 6, only $P_3O_{10}^{5-}$ ions exist and the particles formed will be only by ionic cross-linking. Ionically cross-linked CS beads using TPP, adjusted to pH 5 and 6, have shown to elute lower levels of phosphorus indicating lower degree of phosphorus chain scission (Fwu, Shin, Sung, & Tsung, 1999). In our study, ionically cross-linked CS NPs have been prepared at pH 4.5 and 5.5. CS NPs formed at pH 4.5 were of larger size compared to those formed at pH 5.5. Aggregation studies indicated particles formed at pH 4.5 reduced the size marginally over 24 h (Fig. 1A). However, there was a slight increase in size of particles formed at pH 5.5 over 24 h (Fig. 1B).

Stability of NPs in suspension and freeze dried form were studied at RT and 4 °C for 6 months. The IR spectra of freshly prepared and stored samples confirmed, the CS NPs and catechin loaded CS NPs were stable at both temperatures for a period of 6 months (results not shown).

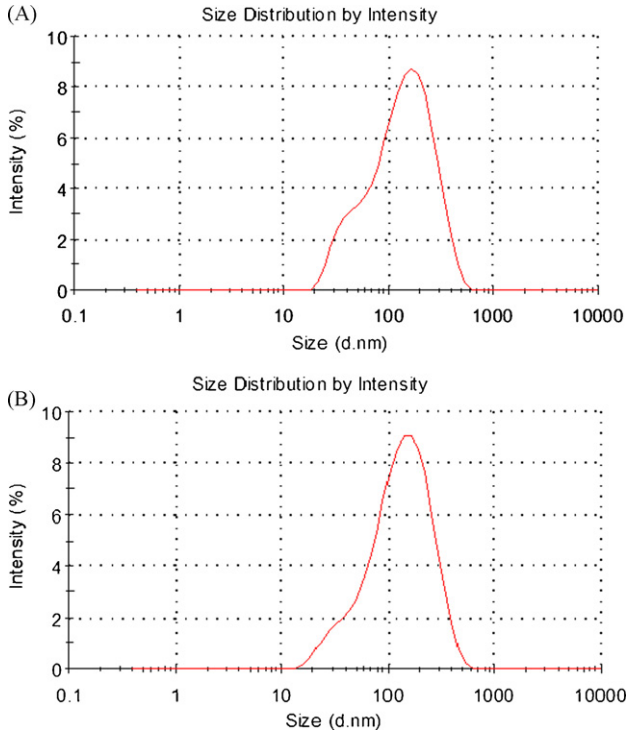

Fig. 2. Particle size distribution by intensity of CS NPs (A) and catechin loaded CS NPs (B) at pH 5.5.

3.2. Characterization studies

Particle size and surface charges are critical determinants for the fate of orally administered NPs. Zeta potential is the surface charge which can influence the particle stability in suspension by the electrostatic repulsion between particles (Qi, Xu, Jiang, Hu, & Zou, 2004). Mean particle size distribution of CS NPs and catechin loaded CS NPs are shown in Fig. 2A and B, respectively. Size of particles formed at pH 4.5 was 168 ± 6 nm with zeta potential 21 ± 4 mV. Particles of size, 110 ± 5 nm and zeta potential of 25 ± 3 mV were observed at pH 5.5. Increase in pH of TPP from 4.5 to 5.5, decreased the particle size and increased zeta potential. The ionic cross-linking process for the formation of CS NPs is pH responsive, providing opportunities to modulate the properties of CS NPs. The differences in zeta potential with change in pH showed; at higher pH more cross-linked particles are formed compared to the lower pH. The particles formed at pH 5.5 were considered for further studies since, smaller size NPs are reported to evade RES and can improve bioadhesion in GIT owing to their size and cationicity.

Zhang and coworkers observed, when commercial LMW CS was used to prepare CS NPs at a concentration of 0.1% (w/w) of CS and TPP (weight ratio of 5:1) produced bimodal particle size distribution of the range 153 and 500 nm (Zhang, Oh, Allen, & Kumacheva, 2004). Likewise, insulin loaded CS NPs prepared at pH 5.3 resulted in particle size range between 243 and 271 nm (Zengshuan, Hock, & Lee, 2002). Mean particle size and zeta potential of catechin loaded CS NPs formed in this study at pH 5.5 were 130 ± 5 nm and 31 ± 2 mV, respectively, with no further significant changes over 24 h (Fig. 2A and B). The zeta potential increased for catechin loaded CS NPs by 5.8 mV in comparison with CS NPs. The catechin in acidic media converts to positively charged flavilium group that could be responsible to increase in the zeta potential. The interactions between phenolic groups of catechin and amino groups of CS (over phosphate group of TPP) may lead to decrease in the cross-linking density (Hu et al., 2008).

Morphological studies showed, CS NPs with or without catechin were nearly spherical in shape with smooth surfaces. TEM images of CS NPs and catechin loaded CS NPs prepared at pH 5.5 are shown in Fig. 3A–C. The result indicates, loading of catechin in CS NPs increased the particle size and it is in agreement with quercetin loaded CS NPs (Yuying, Yan, Kai, Xing, & Guolin, 2008). The size measured in hydrated state is reported to be slightly higher than the size measured by TEM method (Liu, Desai, Chen, & Park, 2005). Result in this study was based on the depiction of the size of dried state (actual diameter) by TEM versus hydrated state (hydrodynamic diameter) measured by light scattering.

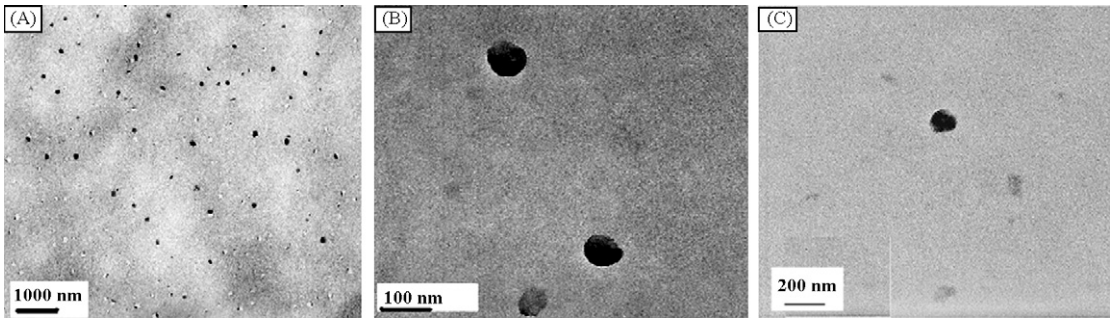

Fig. 3. TEM micrographs of CS NPs: (A) ×10K, (B) ×100K, (C) catechin loaded CS NPs ×50K magnification.

Particle size results from our TEM studies are in the sizes obtained from the dynamic light scattering for catechin loaded CS NPs and CS NPs. Freeze dried CS NPs and catechin loaded CS NPs, redispersed in deionised water with vortexing resulted in nanosize (320–340 nm) irrespective of loading. The increase in the particle size after freeze-drying is found to be common with the unmodified CS particles. This has resulted due to aggregation (Dyer et al., 2002) from the strong inter- and intra-molecular hydrogen bonding which was not possible to breakdown even by sonication (Lee, Powers, & Baney, 2004). The zeta potential of catechin loaded and CS NPs was 24.3 ± 3 mV after freeze-drying indicating slight aggregation tendency of the particles.

FTIR of CS, CS NPs and catechin loaded CS NPs are shown in Figs. 4 and 5. CS showed peaks at 3367 cm^{-1} (ν –OH; –NH), and 1090 cm^{-1} (Fig. 4) (ν C–O–C) (Qi et al., 2004; Wu et al., 2005) which was in agreement to previous reports. Peaks at 1658 cm^{-1} and 1589 cm^{-1} refer to amide I and amino groups as reported earlier (Zhang, Oh et al., 2004). The sharp peaks in CS at 1381 cm^{-1} and 1419 cm^{-1} can be assigned to CH_3 symmetrical deformation mode and peak at 2873 cm^{-1} indicates the –CH stretching vibrations.

After formation, of nanoparticles the shoulder peak at 1658 cm^{-1} reduced dramatically or disappeared and a new peak was formed at 1637 cm^{-1}. The amide II peak at 1589 cm^{-1} in CS was shifted to 1528 cm^{-1} in CS NPs confirming that amino groups were involved in cross-linking by phosphate. FTIR of CS NPs shows an absorption peak at 1217 cm^{-1} which indicates P=O stretching (Qi & Xu, 2004). The peak in the CS at 3367 cm^{-1} widened and shifted to 3100 cm^{-1} in the CS NPs indicating enhanced hydrogen bonding (Fig. 4) (Wu et al., 2005; Xu & Du, 2003). When catechin was loaded in CS NPs, this peak showed more broadening than the CS NPs. This indicates enhanced hydrogen bonding in catechin loaded CS NPs due to interaction of catechin with free amino group of CS (Zhang & Kosaraju, 2007). Peak at 1637 cm^{-1} of CS NPs slightly shifted to a sharper peak at 1625 cm^{-1} in catechin loaded CS NPs may be due to interaction between the hydroxide group of catechin and amino group of CS (Fig. 5). Similar observations were reported by other investigators (Hu et al., 2008). At 1525 cm^{-1} the peak remains unchanged in catechin loaded CS NPs as observed earlier (Zhang & Kosaraju, 2007).

Table 2
DSC thermograms of CS and CS NPs with or without catechin.

Sample	Endotherm I (°C)		Endotherm II (°C)	
	Onset	Endset	Onset	Endset
Pure CS	110.8	121.9	293.1	316.9
CS NPs	113.9	124.7	214.2	227.2
Catechin loaded CS NPs	125.8	138.6	214.5	226.3

Differential scanning calorimetry (DSC) was performed to understand the behavior of CS, CS NPs and catechin loaded CS NPs. Polysaccharides usually have strong affinity for water and in solid state these molecules may have disordered structures. The first endothermic peak corresponds to the evaporation of bound water from the samples (Fig. 6). and CS NPs vary in water holding capacity at different pH conditions. The first endotherm for CS and CS NPs were found to give onset and endset of the transition between 110 and 125 °C (Table 2).

The endotherm of catechin loaded NPs showed a shift to higher temperature between 125 and 139 °C. This may be accounted due to hydrophilic groups incorporated due to catechin. In CS NPs, the

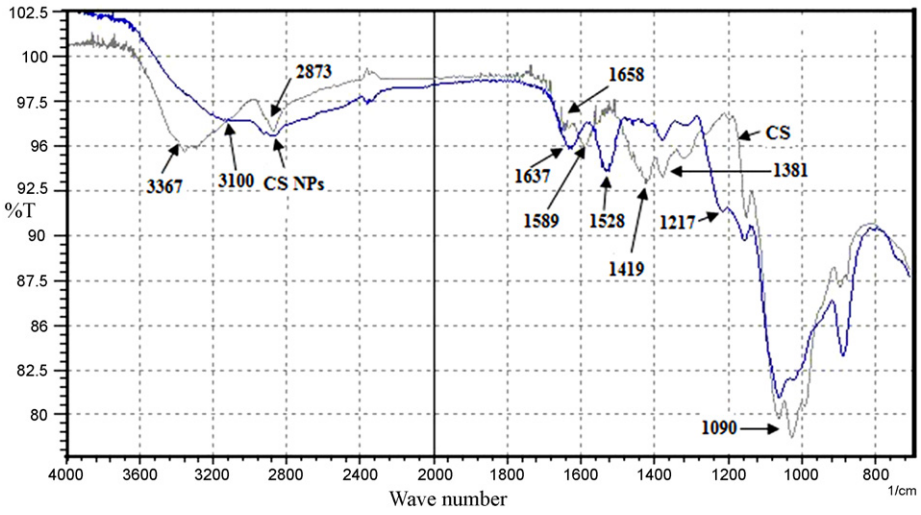

Fig. 4. FTIR spectra of low molecular CS and CS NPs.

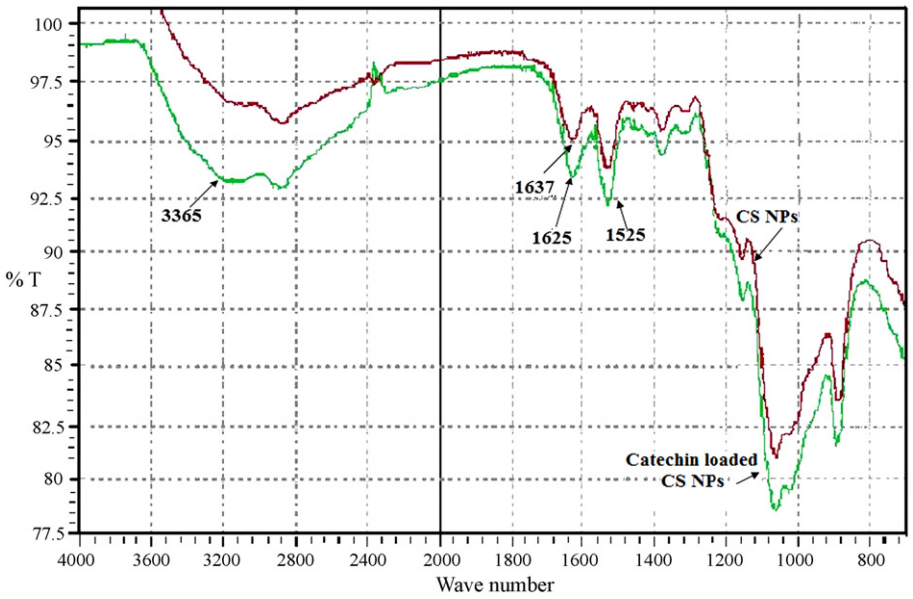

Fig. 5. FTIR spectra of CS NPs and catechin loaded CS NPs.

second endotherm shifted to the lower temperature. Decreased crystallinity indicates change in solid state structure of CS due to cross-linking (Zhang, Oh et al., 2004). Vikas, Kumar, Dinesh, Rakesh, and Ashok (2004) reported similar shifts in DSC plots of CS and CS NPs. The endothermic and exothermic peaks are reported to be shifted to higher temperature when pH of NP preparation was decreased from 5.2 to 4.2. It is suggested that, at lower pH more energy will be required to remove residual water adsorbed to NPs and less energy being released due to the breakage of ionic interactions/thermal decomposition (Sarmento, Ferreira, Veiga, & Ribeiro, 2006). The catechin loaded sample showed the similar shift as the CS NPs, which confirms there are no significant covalent interactions between catechin and CS after encapsulation and cross-linking.

3.3. Determination of entrapment efficiency, loading capacity and percentage yield

The entrapment efficiency of 90 ± 5% and loading capacity 88 ± 4% was observed at pH 5.5, which was higher compared to the yield % (Fig. 7). The low yield of the catechin loaded CS NPs may be accounted due to the competitive interaction between phenolic (OH^-) of catechin and ($P_3O_{10}^{5-}$) groups of TPP for protonated amino groups of CS resulting in low levels of particle formation compared to the CS NPs. Entrapment efficiency and yield at pH 4.5 were observed to be higher than at pH 5.5 exception to loading capacity. When the concentration was 1.44 mg/ml of CS and 0.6 mg/ml of TPP, the encapsulation efficiency was 85% which demonstrated a loading capacity of 7.9% (Wu et al., 2005). The above results suggested that, pH and concentration of TPP may play a significant role during particle formation and also in the encapsulation of bioactives. Previous studies used this method to calculate the entrapment efficiency of the CS particles (Hariharan et al., 2006; Xu, Du, Huang, & Gao, 2003).

Chitosan is susceptible to chitosanase and lysozyme digestion but substrate specificity of the two enzymes is different. Lysozyme attacks near the N-acetylated glucosamine whereas chitosanase degrades the sequence of three consecutive deacetylated units (Mao et al., 2001). In this study, catechin loaded CS NPs were subjected to digestion with chitosanase and lysozyme showed e entrapment efficiency of 60%. Similar results of low entrapment efficiency were obtained in earlier studies (Zhang, Dudhani, Lundin, & Kosaraju, 2009). At lower ratios of CS to TPP, there may be

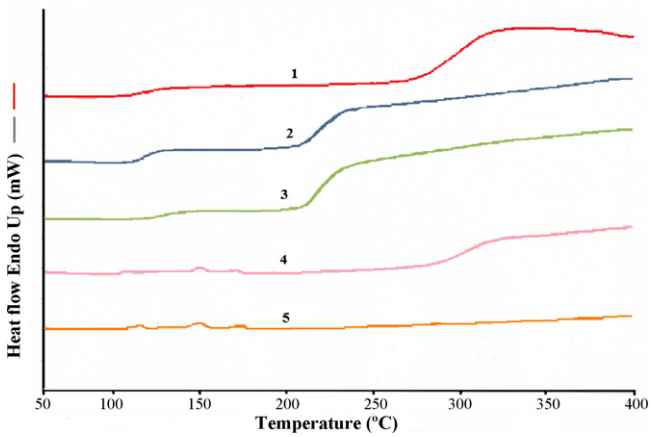

Fig. 6. DSC of (1) pure CS, (2) CS NPs (placebo), (3) catechin loaded CS NPs, (4) physical mixture of CS and catechin and (5) pure catechin.

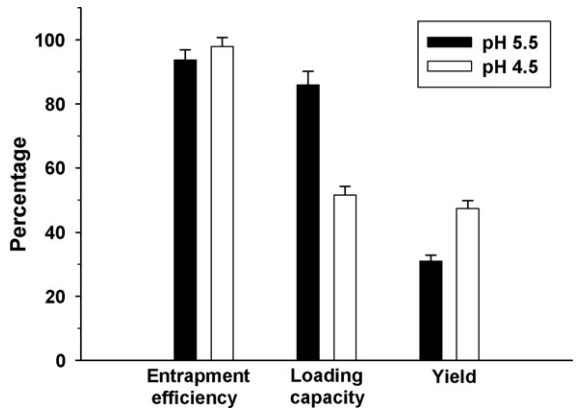

Fig. 7. Comparison of entrapment efficiency, loading capacity and percentage yield of catechin loaded CS NPs prepared at pH 4.5 and pH 5.5.

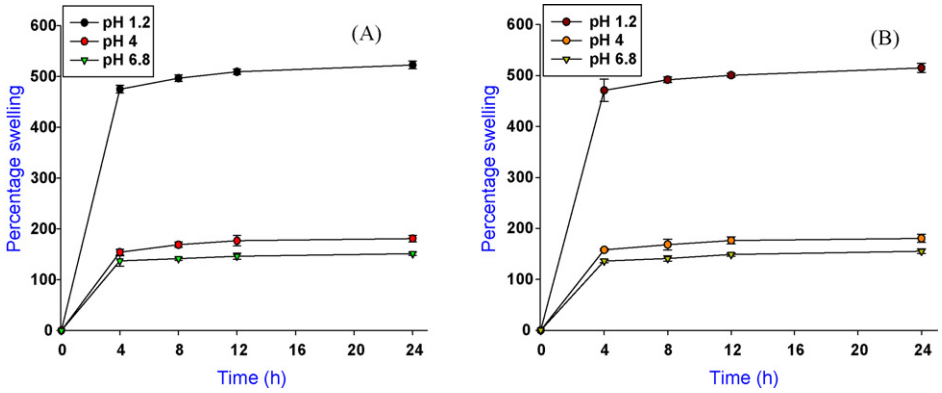

Fig. 8. Swelling of CS NPs (A) and catechin loaded CS NPs (B) prepared at pH different pH conditions.

loosening of the particle matrix that facilitates the extraction of physically entrapped catechin. At higher ratios, there are more amino groups available for catechin to react chemically by forming covalent bonds, which makes it difficult to get extracted even in presence of enzymes. CS NPs encapsulated with DNA/chloroquine and insulin were evaluated by digesting with enzyme mixture and analyzed by spectrophotometry. Association efficiency obtained at pH 5.3 was less than half that measured at pH 6.1 at corresponding insulin concentration. The release of insulin was pH-dependent and recovery was less than 25% even upon disintegration of the chitosan matrix. This demonstrates clearly that the association at pH 5.3 appeared to be based on stronger interactions between insulin and chitosan NPs (Mao et al., 2001).

3.4. Swelling studies

In this study CS NPs demonstrated swelling 474–522%, 154–180%, and 137–151% at pH 1.2, 4 and 6.8, respectively, over 24 h. According to the literature, CS swells in the acidic pH and shows less swelling in the intestinal pH. The electrostatic interaction between anions and CS is controlled by pH and CS/TPP film exhibited pH-dependent swelling (Shu & Zhu, 2002). The swelling of CS NPs was reported to be 669% and 158% at pH 3 and 9, respectively (Bhumkar & Pokharkar, 2006). There was no significant difference observed in the percentage swelling between CS NPs and catechin loaded CS NPs (Fig. 8A and B). Within 4 h, the swelling had almost reached equilibrium in both samples. This may be accounted due to its small particle size. Similar findings were observed with CS/TPP film with a drastic decrease in the swelling ratio from pH 4 to 6.5 followed by a gradual decrease between pH 8 and 10 (Shu & Zhu, 2002). The swelling capacity was reported to decrease from 360% to 320% with increase in TPP concentration from 1% to 2% (Desai & Park, 2005). Higher swelling properties of CS particles could be attributed to their ability to uncoil the polymer to an extended structure and higher molecular weight (Agarwal & Mishra, 1999).

3.5. In vitro release studies

Drug release from NPs and microparticles takes place by several mechanisms including surface erosion, disintegration, diffusion and desorption (Hariharan et al., 2006). The release profile of catechin from CS NPs and the spiked catechin as control are shown in Fig. 9. The complete release of pure catechin (control) was observed within 4 h through the dialysis bag may be due to the low molecular weight of catechin. Catechin loaded CS showed an initial burst release of 2.6% which may be accounted for the bioactive adsorbed to the surface. Over 2 h in simulated gastric fluid (SGF), 15% of catechin was released. There was slow release in the simulated intestinal fluid (SIF) up to 4 h and continued sustained release up to 24 h. The release in SIF was observed as 21%, 22%, 21%, and 32% released at 4 h, 8 h, 12 h and 24 h, respectively. The release in SGF increased from 2.6% to 15% due to the swelling property of the polymer in the acidic pH that was clearly observed in the swelling studies. When the CS NPs were transferred to SIF, the reduced swellability of the polymer has decreased the release. The release of catechin from the nanoparticulate system initially followed zero order kinetics and followed by a sustained release pattern over 24 h. In our study, catechin loaded CS NPs formed at pH 4.5 showed initial release of 5.8% catechin higher than catechin loaded CS NPs formed at pH 5.5 in SGF. This may be attributed to higher entrapment efficiency or catechin adhered to the surface of catechin loaded CS NPs formed at pH 4.5. Cumulative release of catechin increased to 9.9% in SGF over 2 h and overall 7% more catechin was released for pH 4.5 NPs, compared to NPs formed at pH 5.5. Smaller particles formed at pH 5.5 may be contributing to the higher release at acidic pH conditions. In SIF, the release studies of catechin from CS NPs (pH 4.5) demonstrated a sustained release up to initial 4 h followed by rapid release during next 4 h then a steady release (Fig. 9). The results obtained revealed possibility to modulate release rate of catechin by adjusting pH conditions during formation of particles.

Release of the drug from CS matrix has found to follow diffusion in the initial phase followed by degradation of the polymer. Similar results with an initial release of 10% encapsulated BSA were reported with CS NPs and later followed slow release at a constant but different rate (Xu & Du, 2003). Most of the drugs loaded in

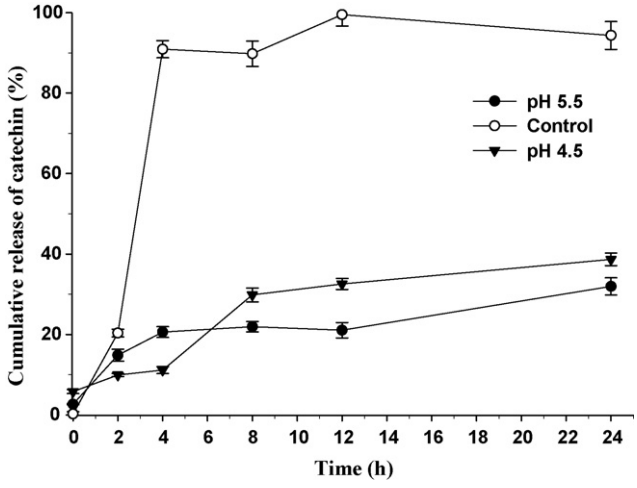

Fig. 9. *In vitro* release of catechin from CS NPs and spiked catechin solution (control).

Table 3
Evaluation of physical/mucoadhesive properties of FITC CS NPs and catechin loaded FITC CS NPs.

Parameters	FITC CS NPs	Catechin loaded FITC CS NPs
FITC in NPs (%)	81 ± 3	75 ± 8
Yield (%)	67 ± 2	39 ± 4
Particle size (%)	124 ± 8	99 ± 5
Zeta potential (mV)	25 ± 4	22 ± 5
Mucoadhesion (%)	32 ± 0.1	40 ± 0.1

NPs or microparticles show biphasic release pattern with an initial burst followed by a sustained release. The process of complexation between polyphenol and CS have been reported to be reversible or irreversible. These are hydrophobic interactions mainly owing to covalent linkages (Zhang & Kosaraju, 2007). Reversible complexation was discussed to occur at two stages, (a) development of non-covalent forces in equilibrium with soluble complexation and (b) after equilibrium changes, the complexes may aggregate and precipitate. Popa, Aelenei, Popa, and Andrei (2000) reported the release of encapsulated polyphenol in acidic and alkaline pH. The release was not significant in acidic media while only 6% was released in the alkaline pH 7.8.

3.6. Mucoadhesive studies

Bioadhesion to mucous membrane in GIT can be described as mucoadhesion. Mucoadhesive polymers improve absorption/bioavailability of drugs with poor absorption characteristics (Kreuter, 1991; Takeuchi, Yamamoto, & Kawashima, 2001; Takeuchi, Yamamoto, Niwa, Hino, & Kawashima, 1996). The mucoadhesion is evaluated widely using fluorescent labeling which offer rapid, sensitive and simple means (Delie, 1998). However, quantification is possible only if the calibration curve is prepared exactly similar to that of the samples. It is important that the fluorescent marker is not dissociated during process, storage and uptake experiments. Recent study investigated adhesion time of the mucoadhesive microspheres on isolated pig intestine with different polymers, like carbopol (CP), chitosan (CS) and methyl cellulose (H). The mucoadhesion and swelling orders ranked in decreasing proportion as CS > CP > H and CP > CS > H, respectively. Moreover, the linear molecule of CS expressed sufficient chain flexibility for interpenetration and entanglement (Harikarnpakdee, Lipipun, Sutanthavibul, & Ritthidej, 2006). Mucoadhesion properties of CS are due to the electrostatic attraction between the positively charged CS and negatively charged sialic acid group of mucin of the intestine (Kawashima, Yamamoto, Takeuchi, & Kuno, 2000). This provides a prolonged contact time between the polymeric system and mucous layer surface to enhance the absorption of the drugs (Soane et al., 1999).

Mucoadhesion of FITC labeled catechin loaded CS NPs and CS NPs were investigated and exhibited 40% and 32% bioadhesion, respectively. Mean particle size distribution and zeta potential of FITC labeled CS NPs and catechin loaded CS NPs were 124 ± 8 nm; 99 ± 5 nm and 24 ± 4 mV; 22 ± 5 mV, respectively, at pH 5.5 (Table 3). The FITC labeled catechin loaded CS NPs had significantly smaller particle size than the FITC labeled CS NPs. The reduced particle size and zeta potential after loading catechin demonstrate chemical interaction between FITC and CS. The percentage labeling efficiency of FITC CS was 3.3 ± 0.1. The percentage of FITC and the percentage yield of FITC labeled CS NPs and catechin loaded CS NPs are shown in Table 3. The FTIR studies of CS and FITC CS confirmed attachment of FITC to protonated amino group of CS (results not shown).

Higher mucoadhesion of the catechin loaded CS NPs may be due to the presence of catechin, in FITC CS NPs which decreased the particle size significantly and zeta potential insignificantly. In case of unlabeled catechin loaded CS NPs, although the particle size was higher, there was corresponding increase in zeta potential. These factors will have an influence on mucoadhesivity of CS NPs in various parts of the GIT leading to controlled/sustained release behavior.

4. Conclusions

CS NPs, a prospective carrier for sustained release of bioactives were prepared by a modified method and characterized. Smaller size and positive zeta potential for catechin loaded CS NPs and CS NPs indicate their potential to provide bioadhesion in the GIT. FTIR analysis of NPs demonstrated the evidence of cross-linking between positively charged amino group and negatively charged phosphate group. Catechin loaded particles demonstrated enhanced hydrogen bonding due to interaction of catechin with unreacted amino groups of CS.

CS NPs had shown an excellent capacity for entrapment of catechin and clear evidence that pH of TPP solution is crucial for the entrapment efficiency, loading capacity and yield. The entrapment efficiency of the particles by the enzymatic breakdown showed 60% and the *in vitro* release was found to be 32% over 24 h. This suggests a greater potential for release of catechin in intestine beyond 24 h owing to the mucoadhesive characteristic. The fate of the particles is unknown when they reach the colon but, there is a possibility for further degradation by the micro flora and release of the remaining catechin. However, mucoadhesive CS NPs is a promising approach for improving the bioavailability of catechin via oral route.

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