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Thiolated pectin: Synthesis, characterization and evaluation as a mucoadhesive polymer

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ABSTRACT

The objective of present study was to enhance mucoadhesive potential of pectin by thiolation. Thiolation of pectin was achieved with esterification with thioglycolic acid. Thiolated pectin was characterized by FTIR, DSC, XRD and SEM analysis. Thiolated pectin was determined to possess 0.60 ± 0.04 mmol of thiol groups/g of polymer by Ellman's method. Comparative evaluation of mucoadhesive property of metformin-loaded ionotropically gelled beads of pectin and thiolated pectin by wash off test using goat intestinal mucosa revealed higher *ex vivo* bioadhesion time of thiolated pectin as compared to pectin. Improved mucoadhesive property of thiolated pectin over the pectin can be attributed to the formation of disulfide bond between mucus and thiolated pectin. *In vitro* release study conducted using phosphate buffer (pH 6.8) revealed a similar release profile of metformin from pectin and thiolated pectin beads. In conclusion, thiolation of pectin improves its mucoadhesive property without affecting the release profile.

1. Introduction

Adhesion is a process of fixing of two surfaces to one another. If the adhesion occurs on mucosal membrane it is termed as mucoadhesion (Andrews, Lavetery, & Jones, 2009). Since the concept of mucoadhesion has been pioneered in the 1980s, numerous attempts like neutralization of ionic polymers, use of linear poly(ethylene glycol) as adhesion promoter for hydrogels, and the development of polymer adhesion conjugates etc. have been taken to improve adhesive properties of polymer. Mucoadhesive polymers based on formation of non-covalent bonds such as hydrogen bonds, van der Waal's forces, and ionic interactions have weak bioadhesion (Bernkop-Schnürch, Kast, & Richter, 2001). Thiolated polymers or thiomers are new generation of mucoadhesive polymers, which mimic natural mechanism of secreted mucus glycoprotein by covalently anchoring in mucus layers by disulfide bonds. Thiol side chains of thiolated polymers interact with cysteine rich subdomains of mucus glycoprotein forming disulfide bonds between mucoadhesive polymer and mucus layer (Bernkop-Schnürch, 2005). Advantages of these polymer are improved mucoadhesive properties, prolonged residence time of delivery systems on mucosal tissue, high cohesive properties which prevent adhesive bond failure, site specific targeting, avoidance of first pass metabolism, reduced dose related side effects as drug is localized at the disease site, and significant cost reduction (Andrews et al., 2009). Thiolation has been successfully done in several polymers like chitosan (Bernkop-Schnürch, Hornof, & Zoidl, 2003), poly(acrylic acid), alginate, polycarbophil etc. (Perera, Hombach, & Bernkop-Schnürch, 2010).

Natural polysaccharides and its derivatives are widely used in pharmaceutical and food industry as biodegradable and biocompatible polymers for a large number of applications such as binding, thickening, emulsifying, gelling agent etc. Pectin is an acidic water soluble, inexpensive, nontoxic heterogenous polysaccharide extracted from citrus peel or apple pomace. It contains linear chains of (1–4) linked α-D-galactouronic acid residues interrupted by some rhamnogalacturonic acid residue and α-L-rhamnopyranose by α-1-2 linkage (Fig. 1). The galactouronic acid of backbone is partially methyl esterified (Sriamornsak, Wattanakorn, Nunthanid, & Puttipipathkachorn, 2008). Pectin is widely used in food technology because of its favourable gelling properties. Due to its biocompatibility, biodegradability and non-toxicity, pectin represents an attractive biopolymer for a variety of pharmaceutical and biomedical applications (Sriamornsak, Thirawong, et al., 2008). It is also used and investigated as a carrier and coating material in pharmaceutical science (Perera et al., 2010). It has bioadhesive properties towards gastrointestinal tissues, which can be used as drug delivery device for target release and optimal drug delivery due to intimacy and duration of contact (Sriamornsak, Sungthongjeen, & Puttipipitkhachorn, 2007).

The present study was designed with the objective to improve the mucoadhesive properties of pectin by synthesizing pectin

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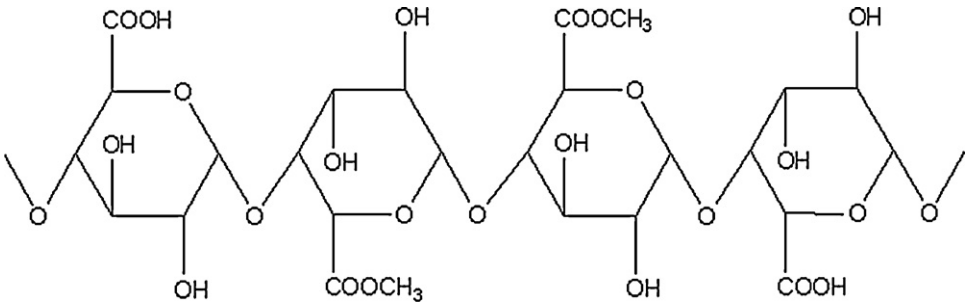

Fig. 1. Structure of pectin.

thioglycolic acid conjugate. Thiolated pectin was characterized by Fourier transform infra-red spectroscopy (FT-IR), differential scanning calorimetry (DSC), X-ray diffraction analysis (XRD), and scanning electron microscopy (SEM). The numbers of thiol groups/g of thiolated pectin were determined by Ellman's method. Thiolated pectin was further explored for mucoadhesive applications by formulating the ionotropically gelled beads employing metformin as model drug. Mucoadhesive properties of pectin and thiolated pectin beads were comparatively evaluated using *ex vivo* bioadhesion study employing freshly excised goat intestinal mucosa. Further, the beads of thiolated pectin and pectin were comparatively evaluated for %entrapment, *in vitro* release and swelling behavior.

2. Experimental

2.1. Materials

Pectin (GENU®pectin (citrus) type USP/100, C.P. Kelco) was gifted by Burzin and Leons Agenturen Pvt. Ltd. (Mumbai, India). Metformin hydrochloride was obtained as gift sample from GMH Lab Pvt. Ltd. (Baddi, India). Calcium chloride (fused) LR, thioglycolic acid, and hydrochloric acid were purchased from SD Fine-Chem Ltd (Mumbai, India). L-Cysteine and Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was procured from Hi-Media Laboratories Pvt Ltd. (Mumbai, India). Freshly excised goat intestine was obtained from the local butcher shop (Hisar, India).

2.2. Synthesis of thiolated pectin

The thiolated pectin was synthesized by the esterification of pectin with thioglycolic acid in the presence of hydrochloric acid. The reaction was carried out with 2 moles of thioglycolic acid for every 1 mole of hydroxyl group in pectin (Dicharry et al., 2006). Pectin (16 g) was dissolved in 70 ml of hot water and was added with 7.59 g of 80% thioglycolic acid and 2 ml of 7 N HCl. These were allowed to react for 150 min at 80 °C. The reaction mixture was poured in 500 ml of methanol. White precipitates of thiolated pectin so obtained were washed twice with methanol and dried at room temperature.

2.3. Characterization of thiolated pectin

2.3.1. Determination of thiol group content

The degree of thiol group substitution was determined by quantifying the amount of thiol group on thiolated pectin and pectin (control) by Ellman's method (Bernkop-Schnürch et al., 2003).

An accurately weighed 50 mg of thiolated pectin or pectin (control) were dissolved in 25 ml of distilled water. An aliquot of 2.5 ml of the polymer solution diluted with 2.5 ml of 0.5 M phosphate buffer (pH 8.0) was allowed to react with 5 ml of Ellman's reagent (DTNB, 0.03% w/v in 0.5 M phosphate buffer pH 8.0) for 2 h at room temperature, followed by measurement of absorbance of the reaction mixture at 450 nm. The number of thiol groups in the polymer were calculated using the standard curve prepared by reacting pectin solution containing varying amount of L-cysteine with Ellman's reagent as explained above

2.3.2. Fourier transform infra-red spectroscopy (FT-IR)

Pectin and thiolated pectin were subjected to FT-IR spectroscopy in a Fourier–transform infrared spectrophotometer (Perkin Elmer spectrum) in range of 4000–400 cm^{-1} as KBr pellets.

2.3.3. Differential scanning calorimetry (DSC)

DSC thermograms of pectin and thiolated pectin were recorded using a differential scanning calorimeter (Q_{10} TA systems, USA). About 5 mg of sample were crimped in a standard aluminium pan and heated in a temperature range of 40 °C to 250 °C at a heating rate of 10 °C per minute in nitrogen atmosphere.

2.3.4. X-ray diffraction analysis (XRD)

The X-ray diffractometry was carried out to investigate crystallinity of the pectin and thiolated pectin. Study was carried out using an X-ray diffractometer (Miniflex 2, Rigaku, Japan). The pectin and thiolated pectin in powder form were scanned from 0° to 80° diffraction angle (2θ) range under the following measurement conditions: source, nickel filtered Cu-Kα radiation; voltage 35 kV; current 25 mA; scan speed 0.05 min^{-1}.

2.3.5. Scanning electron microscopy (SEM)

The shape and surface morphology of pectin, thiolated pectin and drug loaded pectin and thiolated pectin beads were investigated using scanning electron microscope (JEOL, JSM-6100). The samples were coated with gold and mounted on a sample holder. The electron micrographs were taken at an accelerating voltage of 5 kV.

2.4. Preparation of pectin and thiolated beads

Metformin-loaded beads of pectin and thiolated pectin were prepared by ionotropic gelation method using calcium chloride as cross-linking agent (Sriamornsak, Nunthanid, Cheewatanakornkool, & Manchun, 2010; Sriamornsak et al., 2007). An aqueous solution of pectin or thiolated pectin (5%, w/v) containing metformin (0.5%, w/v) was extruded through #18G needle into aqueous solution of calcium chloride (3%, w/v) at room temperature. The gelled beads were allowed to cross-link for 20 min followed by washing with distilled water and drying at 40 °C for two days.

2.5. Evaluation of pectin and thiolated pectin beads

2.5.1. Swelling study

Accurately weighed beads were placed in petridish containing 20 ml of phosphate buffer (pH 6.8) at 37 ± 0.5 °C in incubator (Narang Scientific Works Pvt. Ltd.). Beads were removed at regular interval of time, blotted with filter paper and weighed. Swelling (%) was calculated using the following equation.

$$\%\text{swelling} = \frac{W_e - W_o}{W_e} \times 100 \quad (1)$$

where W_e = weight of beads after equilibrium swelling and W_o = weight of dry beads.

2.5.2. Mucoadhesion study

The *ex vivo* bioadhesion of beads formulation was determined by wash-off test (Lehr, Bowstra, Tukker, & Junginer, 1990). A freshly excised goat intestine was obtained from a local butcher house (Hisar, India) within an hour of slaughter and transported to laboratory in isotonic saline solution. It was cleaned by washing with isotonic saline solution. The intestinal mucosal membrane was pasted on glass slide with cyanoacrylate glue. About 100 beads of pectin and thiolated pectin were adhered to intestinal mucosal tissue by applying light force with fingertip for 30 sec. The glass slide was hung on to arm of USP tablet disintegrating machine which was suspended in 900 ml of phosphate buffer (pH 6.8) at 37 ± 0.5 °C and tissue specimen was given slow, regular up and down movement by operating the USP tablet disintegrating test machine. The number of beads adhering to tissue were counted at regular intervals up to 5 h.

2.5.3. Entrapment efficiency

Entrapment efficiency is the percentage of actual mass of drug encapsulated in the polymeric matrix, related to initial amount of loaded drug.

$$\%\text{entrapment efficiency} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100 \quad (2)$$

For the theoretical drug loading it was assumed that entire drug gets encapsulated in beads. For actual drug loading an accurately weighed 25 mg of beads were taken, grounded in mortar pestle and then sonicated in 100 ml of phosphate buffer (pH 6.8) for 30 min, filtered through 0.45 μm syringe filter and diluted appropriately. The content of metformin hydrochloride in the sample was determined spectrophotometrically by measuring the absorbance at 233 nm in UV–vis spectrophotometer (Cary 5000, Varian Australia).

2.5.4. In vitro drug release studies

An accurately weighed 100 mg of beads were taken. The release rate of drug from these beads was determined using USP type II dissolution apparatus (TDT-08L, Electrolab, India). The beads were enclosed in the muslin cloth and the cloth was tied with the paddle. The paddle was then immersed in the phosphate buffer (pH 6.8) maintained at 37 ± 0.5 °C and was rotated at the speed of 50 rpm (Das & Maurya, 2008). Sample aliquots of 5 ml were withdrawn at regular intervals and the withdrawn sample was estimated for its drug content by measuring absorbance at 233 nm in UV–vis spectrophotometer (Cary 5000, Varian Australia).

3. Results and discussion

The covalent attachment of pectin to thioglycolic acid was achieved by ester bonds formation between hydroxyl group of galacturonic acid moieties of pectin and carboxyl group of thioglycolic acid (Fig. 2). After being ground in a mortar, product appeared as off-white odorless powder, soluble in water. The average yield of this synthesis amounted to 48% of the utilized amount of pectin. Thioglycolic acid is soluble in water and methanol. Precipitation with methanol from an aqueous solution and subsequent washing by keeping the precipitate overnight was found to be sufficient purification method for thiolated pectin. Thiolated pectin was found to contain 0.60 ± 0.04 mmol of thiol groups/g as determined by quantifying the amount of thiol group by Ellman's method.

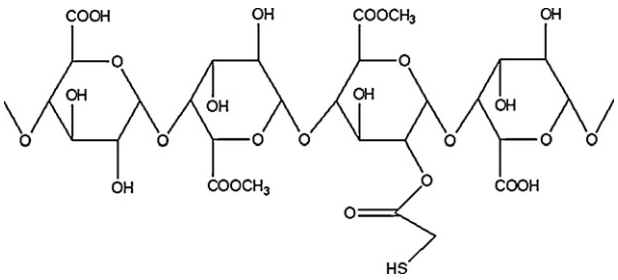

Fig. 2. Structure of thiolated pectin.

Fig. 3 exhibits the FT-IR spectrum of pectin and thiolated pectin in the frequency region from 4000 to 500 cm^{-1}. The IR data of thiolated pectin was as follows: 2573 (SH stretch, weak band, of mercaptans), 1739 (C=O stretch of ester), 3247 (OH stretch, broad, of carboxylic group), 1238 (C–O stretch of carboxylic group), 3516, 3551 (OH band of aliphatic alcohol), 1094 (C–O stretch of primary alcohol). The IR data of pectin was as follows: 1736(C=O stretch of ester), 3328(OH stretch of carboxylic group), 1245 (C–O stretch of carboxylic group), 3445 (OH band of aliphatic alcohol), 1091 (C–O stretch of primary alcohol). Presence of –SH stretch at 2573 and more intensity of C=O stretch of ester confirms the formation of thiolated pectin.

Fig. 4 shows the DSC thermograms of pectin and thiolated pectin. The DSC curve of pectin shows a broad endotherm at

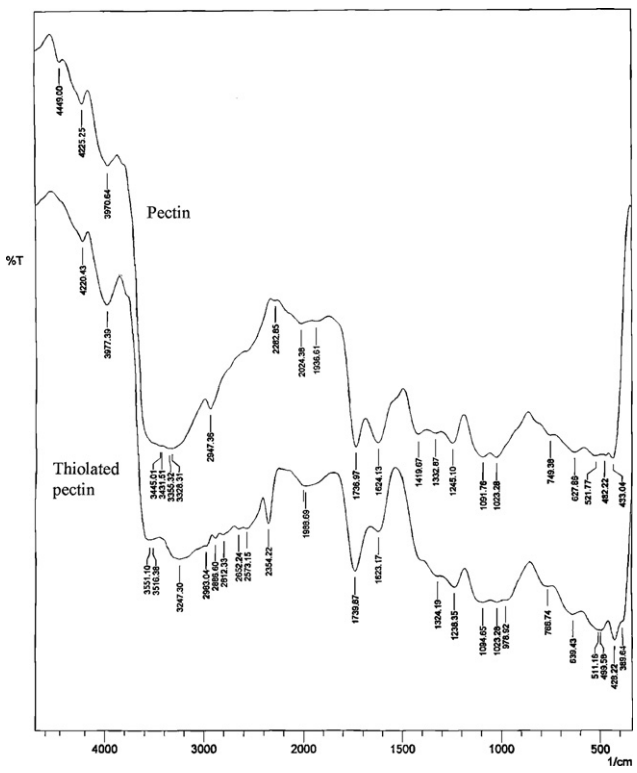

Fig. 3. FTIR spectra of pectin and thiolated pectin.

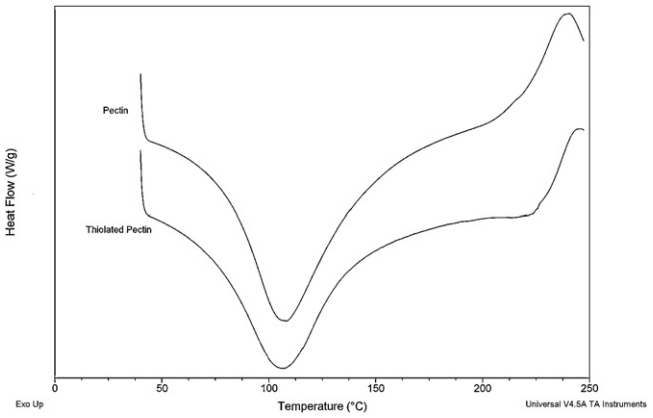

Fig. 4. DSC thermograms of pectin and thiolated pectin.

108.21 °C with heat of fusion of 498.51 J/g. DSC thermogram of thiolated pectin shows an endotherm at 106.97 °C with heat of fusion of 402.67 J/g. Thus, a decrease in the endothermic transition temperature and heat of fusion of pectin was observed on thiolation.

Fig. 5 displays the X-ray diffraction spectra of pectin and thiolated pectin. X-ray diffractogram of pectin is typical of amorphous material with characteristic peaks appearing at 13.56° and 22.56° (2θ) while the diffractogram of thiolated pectin shows characteristic peak at 14.66° and 21.72° (2θ). The peak intensity of thiolated pectin is slightly greater than the pectin.

Fig. 6(a–f) shows the shape and surface morphology of pectin, thiolated pectin, drug loaded pectin beads and thiolated pectin beads as examined under a scanning electron microscope. The shape of pectin and thiolated pectin particles was found to be polyhedral. A close examination of surface morphology reveals that surface of thiolated pectin was rougher and carried large number of grooves in comparison to pectin. Fig. 6(e and f) shows the surface morphology of drug loaded pectin and thiolated pectin beads formed by ionotropic gelation method. The beads of pectin were spherical in shape and had smooth surface while beads of thiolated pectin were also spherical but had rough and grooved surface.

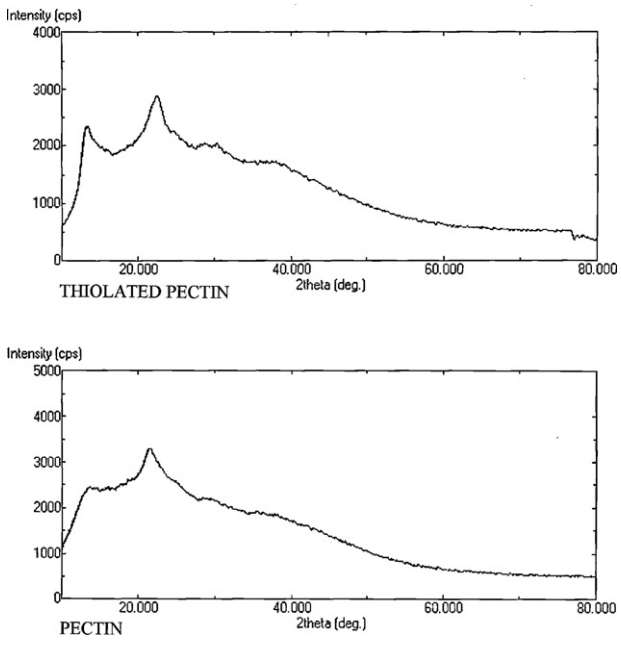

Fig. 5. XRD spectra of pectin and thiolated pectin.

Table 1
Ex vivo bioadhesion and swelling study conducted using metformin-loaded pectin and thiolated pectin beads.

Time (min)	Pectin		Thiolated pectin	
	Swelling (%)	Mucoadhesion (%)	Swelling (%)	Mucoadhesion (%)
0	0	100	0	100
15	–	94	–	100
30	53.7	88	28.4	100
60	66.9	80	32.6	91
90	–	54	–	85
120	67.4	34	44.6	82
150	–	22	–	80
180	51.9	0	53.2	37
210		0	–	34
240		0	52	29
270		0	–	23
300		0	50.7	16

– represents values not determined.

Calcium ion-induced gelation of pectin results from the specific and strong interactions between calcium ions and blocks of galacturonic acid residues of pectin. An egg-box model for interaction between calcium ion and polygalacturonate chains has been described. According to this model, calcium ion induces a chain–chain association between the two-parallel or antiparallel polygalacturonate helical chains forming junction zones. These junction zones comprise of calcium ions coordinating with a pair of galacturonate chains of pectin (Braccini & Perez, 2001).

In the present study, metformin was used as the model drug, under the conditions of experiment the interaction between the basic amino groups and acidic carboxylate groups of pectin appear to be remote. Even if some interaction takes place between the two, it will result in increasing acidity on the carboxylate ion of the galacturonic acid residues leading to much stronger interaction with the divalent cation (Ca^{2+}). Moreover, earlier studies reported amidated pectin to form more compact Ca-pectinate network than the non-amidated pectins (Das, Ng, & Ho, 2010; Wakerly, Fell, Attwood, & Perkins, 1997).

Calcium ion induced gelation of pectin has been used frequently for preparing ionotropically gelled beads of pectin (Aydin & Akbug, 1996; Sriamornsak, 1999). During earlier studies ionotropically gelled beads of pectin have been explored for colon targeting (Chambin, Dupuis, Champion, Voilley, & Pourcelot, 2006; Das & Ng, 2010) and as mucoadhesive formulation (Hagesaether, Bye, & Sande, 2008). Thus, thiolated pectin was formulated as beads for exploiting its mucoadhesive application employing metformin as a model drug. The beads of pectin and thiolated pectin formed by ionotropic gelation method were found to entrap 74.60% and 80.46% of metformin respectively.

Table 1 summarizes the results of *ex vivo* bioadhesion and swelling studies conducted using metformin-loaded pectin and thiolated pectin beads. It is evident from the results that the beads formulated using pectin were washed away in 2.5 h while thiolated pectin beads were still adhering until 5 h of study. The mucoadhesive property of pectin can be attributed to the presence of–OH groups, which form hydrogen bonds with mucus molecules. Pectin also forms non-covalent bonds like van der Waal's forces or ionic interactions, resulting in weak mucoadhesion. The superior mucoadhesive property of thiolated pectin can be attributed to the formation of disulfide bonds between the –SH groups of thiolated pectin and mucus.

Swelling behavior of the mucoadhesive polymers affects its adhesive and cohesive properties. Mucoadhesive beads are anticipated to take up water from the underlying mucosal tissue by absorbing, swelling and capillary effects, leading to considerable stronger adhesion (Dhaliwal, Jain, Singh, & Tiwary, 2008). The

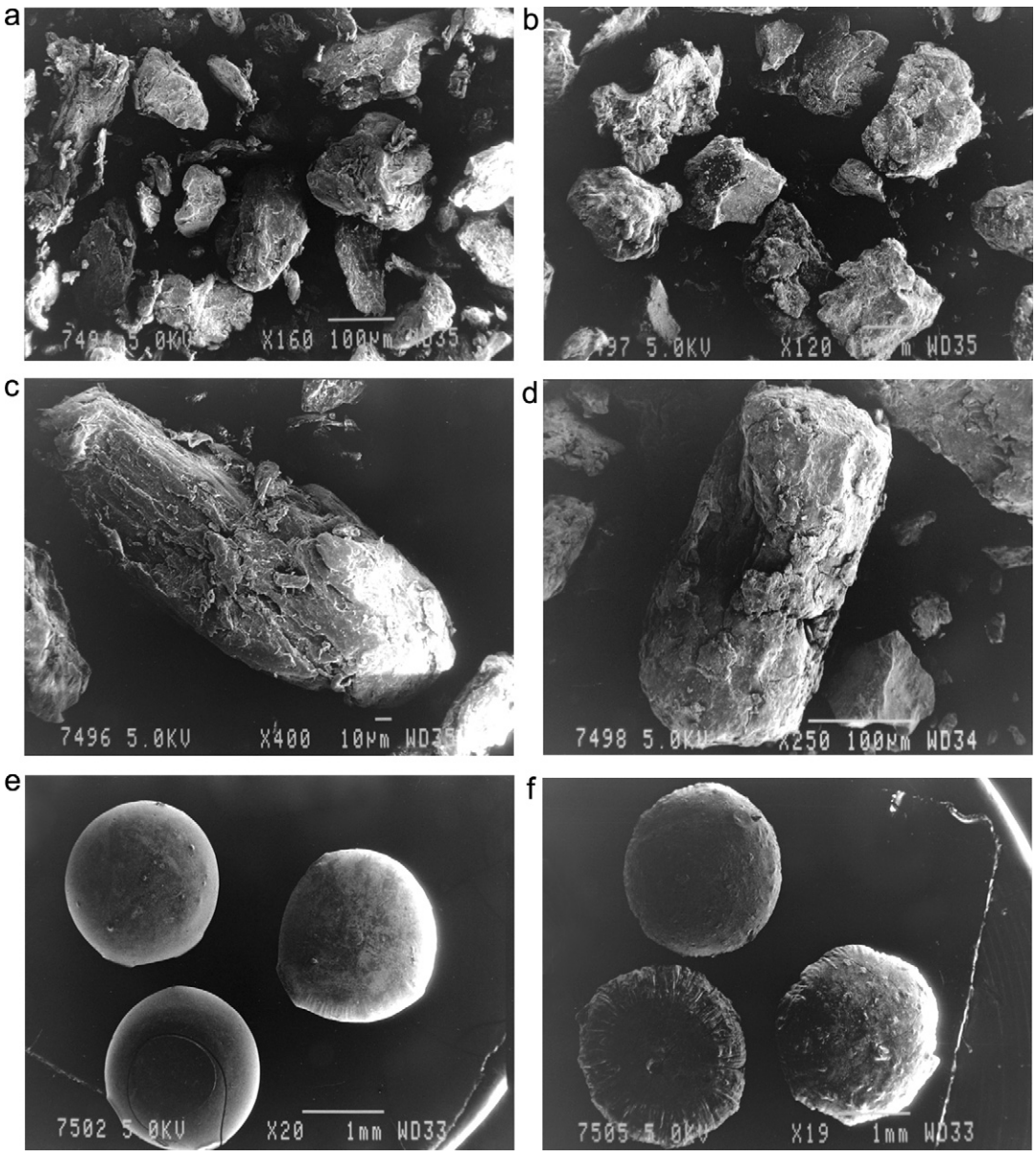

Fig. 6. SEM photomicrographs of (a) pectin, (b) thiolated pectin, (c) surface of pectin, (d) surface of thiolated pectin, (e) drug loaded pectin beads, (f) drug loaded thiolated pectin beads.

results of swelling study revealed that metformin-loaded beads of pectin hydrated quickly, with a %swelling of 66.9 in the first hour compared to the beads formulated using thiolated pectin which showed a %swelling of 28.4. Further the beads of pectin also eroded at a faster rate than the thiolated pectin beads. Pectin, a hydrophilic macromolecule is a 'first-generation' mucoadhesive, whose mucoadhesive properties depend upon the presence of hydrogen bond forming groups. First-generation mucoadhesives are activated by moistening and they adhere non-specifically to many surfaces. However, their overhydration results in the formation of loosely bound slippery mucilage leading to their removal from the surfaces. The faster swelling and erosion of the pectin beads could be one of the reasons for their shorter biaodhesion time. Thus, the results of swelling study support our findings of *ex vivo* bioadhesion study.

Fig. 7 displays the *in vitro* release profile of metformin from pectin and thiolated pectin beads. It can be observed from the results that release of metformin from pectin and thiolated pectin beads is almost similar with both the formulations releasing 50% of the drug in 24 h. Thus, thiolation of pectin provided us with the

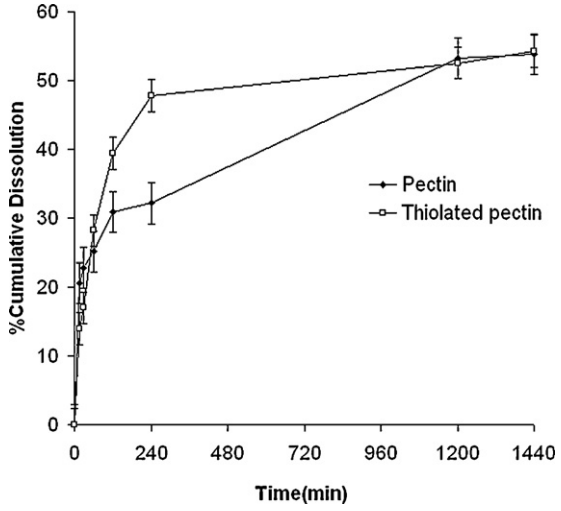

Fig. 7. *In vitro* release profile of metformin from pectin and thiolated pectin beads at different time intervals.

means of enhancing the bioadhesion time without affecting the release rate.

4. Conclusion

On the basis of this study, it can be concluded that modification of pectin by thiolation employing esterification with thioglycollic acid provides with the means of improving the mucoadhesive potential of pectin. Thiolation of pectin at the level of degree of thiolation of 0.6 mmol/g did not affect its calcium-ion induced ionotropic gelation. Similar release profiles of metformin from the pectin and thiolated pectin beads indicate the potential usefulness of thiolated pectin in formulating the mucoadhesive extended-release delivery system for metformin. However, further studies *in vivo* are needed to comment more in this respect.

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