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Pectin–cysteine conjugate: synthesis and in-vitro evaluation of its potential for drug delivery

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Abstract

This study was aimed at improving certain properties of pectin by introduction of thiol moieties on the polymer. Thiolated pectin was synthesized by covalent attachment of cysteine. Pectin–cysteine conjugate was evaluated for its ability to be degraded by pectinolytic enzyme. The toxicity profile of the thiolated polymer in Caco-2-cells, its permeation enhancing effect and its mucoadhesive and swelling properties were studied. Moreover insulin-loaded hydrogel beads of the new polymer were examined for their stability in simulated gastrointestinal conditions and their drug release profile. The new polymer displayed 892.27 ± 68.68 μmol thiol groups immobilized per g polymer, and proved to have retained its biodegradability, upon addition of Pectinex Ultra SPL in-vitro, determined by viscosity measurements and titration method. Pectin–cysteine showed no severe toxicity in Caco-2 cells, as tested by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. Moreover, the synthesized polymer exhibited a relative permeation enhancement ratio of 1.61 for sodium fluorescein, compared to unmodified pectin. Pectin–cysteine conjugate exhibited approximately 5-fold increased in in-vitro adhesion duration and significantly improved cohesive properties. Zinc pectin–cysteine beads showed improved stability in simulated gastrointestinal media; however, insulin release from these beads followed the same profile as unmodified zinc pectinate beads. Due to favourable safety and biodegradability profile, and improved cohesive and permeation-enhancing properties, pectin–cysteine might be a promising excipient in various transmucosal drug delivery systems.

Introduction

Pectin is a naturally occurring, biodegradable polymer. It is predominantly a linear polymer of α-(1–4)-linked D-polygalacturonic acid residues. Pectin has been extensively studied as a matrix polymer and coating material for oral, buccal, vaginal, transdermal and ophthalmic drug delivery systems (Giunchedi 1999; Martin et al 2000; Shen & Mitragotri 2002; Musabayane et al 2003; Sande 2005; Valenta 2005). It can control the drug release via pH- and time-dependent mechanisms in pharmaceutical dosage forms. Furthermore, pectin can be also broken down by pectinase enzymes produced by anaerobic bacteria in the colon, controlling the drug release by this mechanism. However, due to very high water solubility, which leads to rapid hydration, swelling and erosion, pectin is shown not to be capable of controlling drug release efficiently in different dosage forms. This drawback has thus far limited application of pectin in drug delivery systems. Hence, less water-soluble forms of pectin should be used in this regard (Burgalassi 1996; Sinha & Kumria 2001). Some measures have been taken to overcome this problem. Modified pectins or their combination with different polymers have been suggested for delivery of drugs via various administration routes. Semdé et al (2002) synthesized epichlorohydrin cross-linked pectins with decreased water solubility. Another approach is utilizing the ability of low-methoxy pectin to form gel beads, with reduced solubility and swelling in the presence of many divalent cations (Wellner et al 1998). Moreover, introduction of amide groups into low-methoxy pectin molecule tends to make the pectin less hydrophilic (CP Kelco information sheet; Wakerly et al 1997). However, these measures have not been sufficient. For instance, in the case of orally administered pectin tablet or

beads, they need to be coated or mixed with other polymers if they are to be administered for colon-specific drug delivery (Mura et al 2003; Atyabi et al 2005). These issues emphasize the need for further modification of pectin to obtain the desired pharmaceutical profiles.

Thiolation is known to improve some properties of polymers. The so-called thiomers are mucoadhesive polymers that possess thiol-bearing side chains. In contrast to many well-established mucoadhesive polymers, these novel polymers are capable of forming covalent disulfide bonds with the cysteine-rich subdomains of mucus gel layer on the mucosa (Bernkop-Schnürch 2005). Hence, the introduction of the thiol moieties into pectin was carried out to improve its mucoadhesive properties. Furthermore, thiomers have been shown to possess permeation-enhancing properties (Bernkop-Schnürch et al 2004a, b). On the other hand, the thiol groups on thiomers can be, to some extent, oxidized under very mild conditions to form intermolecular covalent bonds and, as a result, modulate water absorption, swelling behaviour and erosion of pectin, which leads to improved stability of its formulations.

Therefore, the objective of this study was to synthesize a new thiolated pectin derivative to improve specific properties of this polymer for improved in-vivo administration. The new polymer was evaluated for biodegradability and cell toxicity. Its mucoadhesive and permeation-enhancing properties were also assessed. Furthermore, the polymer's potential as a multiparticulate bead dosage form for oral drug delivery was investigated.

Materials and Methods

Materials

Low-methoxy amidated pectin type GENU pectin LM-104 AS-FS (Pec) (D.M. = 28%, D.A. = 20%) was purchased from CPKelco (Denmark). Pectinex Ultra SPL (pectinase from *Aspergillus aculeatus*, activity 26000 PG mL^{-1} at pH 3.5), L-cysteine hydrochloride monohydrate (Cys), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), Ellman's reagent (DTNB, 5,5´-dithiobis(2-nitrobenzoic acid)), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), insulin from bovine pancreas (27 USP units/mg), zinc acetate, sodium thiosulfate, reduced L-glutathione (GSH) and sodium fluorescein (NaFlu) were obtained from Sigma (Austria). Lactate dehydrogenase (LDH) cell toxicity assay kit was purchased from Roche (Switzerland). Polycarbophil–cysteine conjugate (PCP-Cys) was synthesized by our research group as previously described (Bernkop-Schnürch et al 1999). All other chemicals used were of reagent grade.

Synthesis of pectin–cysteine conjugate

The covalent attachment of Cys to Pec was achieved by formation of amide bonds between the primary amino groups of the amino acid and carboxylic acid groups of the polymer by a method previously developed by our research group for alginate (Figure 1) (Bernkop-Schnürch et al 2001). Briefly,

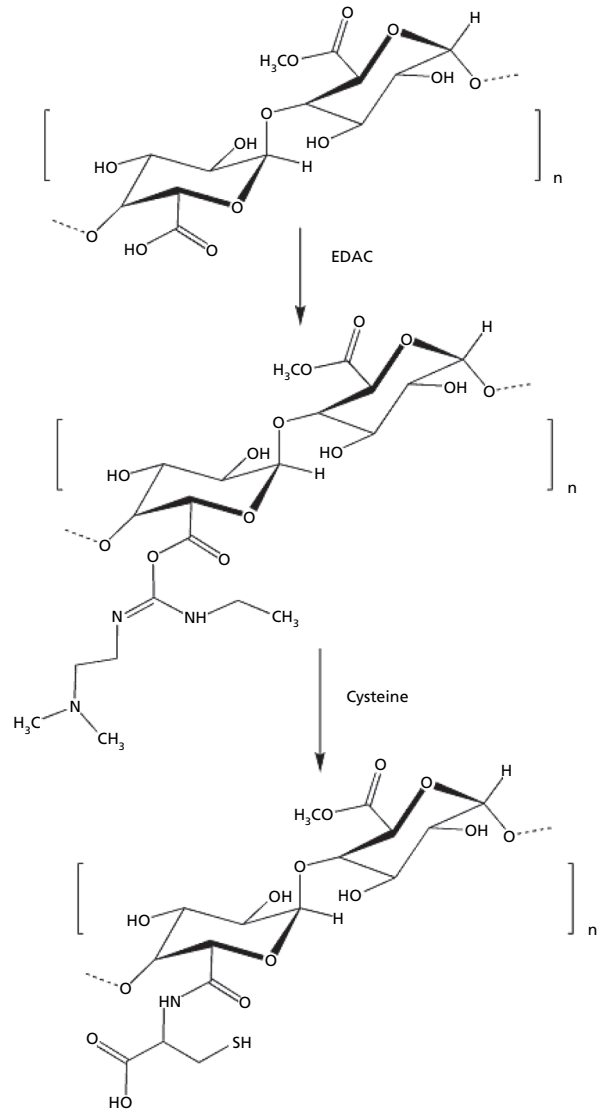

Figure 1 Schematic synthesis of pectin–cysteine conjugate. The formation of amide bonds between the primary amino acid of cysteine and the carboxylic acid group of the polymer was mediated by a carbodiimide (EDAC).

2 g of Pec was hydrated in de-mineralized water. The carboxylic acid moieties of the polymer were activated by the addition of EDAC (50 mM) for 45 min. Thereafter, Cys solution (pH 4, 4 g, Pec–Cys weight ratio of 1:2) was added and the final volume was made to 200 mL and stirred for to 2 h. Then the pH was raised to 5.0 and the reaction was allowed to proceed for 75 min. The resulting Pec-Cys conjugate was isolated by dialyzing in zero light conditions at 10 °C. The Pec-Cys conjugate was dialysed against 1 mM HCl (pH 4) (× 1), 1 mM HCl (pH 4) containing 1% NaCl (× 2) and 1 mM HCl (pH 4.0) for an extended period of time to isolate pure conjugate. Unmodified Pec control was prepared in the same way except that EDAC was omitted. Samples were lyophilized by drying frozen aqueous polymer solutions at −50°C and 0.01 mbar (Lyolab B; Inula, Austria).

Determination of immobilised thiol groups and extent of disulfide bond formation

The amount of immobilised thiol groups was determined with Ellman's reagent according to a method described previously (Bernkop-Schnürch et al 1999). The extent of disuldfide bond formation was determined after reduction with $NaBH_4$ and addition of Ellman's reagent as described by Habeeb (1973).

Determination of unbound cysteine

Quantification of remaining unbound Cys in the conjugate was achieved by utilizing TNBS (Bernkop-Schnürch et al 2003). Pec-Cys (1 mg) was hydrated in 500 μL of 0.5% NaCl solution. After addition of 500 μL of TNBS solution (2% v/v in 8% $NaHCO_3$ solution), the sample was incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a plate reader (Fluostar Galaxy; BMG Labtech, USA). The amount of unconjugated Cys was calculated using a standard curve.

In-vitro evaluation of biodegradability

Viscosity measurement

Pec or Pec-Cys solutions (1% w/v, pH 4) were used for enzymatic biodegradation studies. Pec-Cys was also tested after oxidation, by addition of 10 μL of hydrogen peroxide (3%) to 5 mL of gel solution. The viscosity of 700-μL volumes was measured before and after addition of Pectinex Ultra SPL (6 μL/5 mL), at predetermined time points at 25 ± 0.5°C and shear rate of 10 s^{-1}, using a cone and plate viscometer (Rheolab MC1; Paar Physica, Austria). The initial viscosities of the polymers differed. Therefore, to be able to compare the trend in viscosity changes, the term comparative viscosity (η_c) was coined and used throughout the study. η_c was calculated by the following equation:

$$\eta_c(\%) = (\eta_t / \eta_o) \times 100 \quad (1)$$

where, η_o is initial viscosity of polymeric gel and η_t is the viscosity at time t.

Enzymatic assay by titration method

This method was used as a complementary assay to viscosity measurements to verify the biodegradability of Pec-Cys. In this assay, pectinase catalyses the hydrolysis of pectin molecules to galacturonic acid. In the presence of iodine, any galacturonic acid that is produced will be oxidized. Excess iodine (which is not reduced) is in turn titrated with sodium thiosulfate (Sigma product information sheet for Pectinex Ultra SPL) in the next stage of the experiment.

In brief, 100 μL of diluted solution of Pectinex Ultra SPL (30 μL mL^{-1}) in ice-cold distilled water was added to 5 mL of Pec or Pec-Cys test solutions (0.5%, pH 4). Pec-Cys was also tested upon oxidation by addition of 10 μL of hydrogen peroxide (3%) to 5 mL of gel solution. Blank and test solutions were incubated at 25°C for exactly 5 min. Thereafter, 5 mL of I_2–KI solution (50 mM I_2 with 200 mM KI) and 1 mL of Na_2CO_3 solution (1 M) were added, mixed by swirling and stored for 20 min in the dark. Two millilitres of H_2SO_4 (2 N) was then added. The solutions were titrated with $N_2S_2O_3$ (100 mM) to obtain a light yellow colour. Consequently, 1 drop of starch indicator was added to obtain a blue colour. The titration was continued until solutions became colourless. The final volume of $N_2S_2O_3$ reagent was recorded. The difference of the consumed volume in blank and test samples (V_e) is an indirect indicator of galacturonic acid production and hence enzymatic biodegradation of the polymer:

$$V_e = V_b - V_t \quad (2)$$

where, V_b and V_t are defined as the volume (mL) to titrate the excess iodine in blank and test samples, respectively.

Cell cultures

Caco-2 cells were grown in 75-cm^2 culture flasks, at 37°C and 5% CO_2 atmosphere in MEM growth medium supplemented with 20% (v/v) fetal bovine serum, 2 mM L-glutamine, 0.22% $NaHCO_3$, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). The medium was changed every day and the cells were trypsinized twice per week. The splitting rate was 1–6. The cells with passage number 71–80 were used in these studies.

Cytotoxicity studies

MTT assay

MTT is a tetrazolium salt that is cleaved to a dark blue product by mitochondrial dehydrogenases in living cells (Vihola et al 2005). The Caco-2 cells were cultured in 6-well plates for 24 h. Subsequently, culture medium was replaced with 2 mL of HBSS (Hanks Balanced Salt Solution)–HEPES, and Pec or Pec-Cys (0.25 or 0.5% w/v) was added to the wells. As a negative control, polymer was not added to some of the wells. The cells were further incubated at 37°C for either 3 or 16 h. Thereafter, the polymers were carefully removed by aspiration; 1 mL of MTT solution (0.5 mg mL^{-1} in MEM without phenol red and FCS) was added and incubated for another 2 h. At this stage, the MTT solution was removed and the formazan crystals that were produced were solubilized in 1 mL of cold acidic isopropanol before quantifying the colour of reaction product using a spectrometer (DU Series 600; Beckman Instruments Inc., USA) at 570 and 650 nm. Cell viability (as a percent of the negative control) was calculated from the absorbance values.

Lactate dehydrogenase cytotoxicity assay

LDH is a cytosolic enzyme that is not normally secreted outside of the cell. However, it leaks into the culture medium upon damage to cell membranes (Vihola et al 2005). LDH activity was measured in culture supernatants after either 3- or 16-h exposure of cells to Pec or Pec-Cys (0.25 or 0.5% w/v) using a cytotoxicity assay kit. Blank HBSS and 2% Triton X-100 were used as negative and positive controls, respectively. Absorbance values were measured at 490 nm with a plate reader. Cytotoxicity of the samples was calculated as follows:

$$\text{Cytotoxicity}\ (\%) = (A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}}) \times 100 \quad (3)$$

where A_{sample} is the absorbance value for the cells that were treated with polymer samples, $A_{\text{negative control}}$ is the absorbance value for the spontaneous release of LDH in blank HBSS buffer and $A_{\text{positive control}}$ is the absorbance value for maximum LDH release in lysed cells with Triton X-100.

Transport experiments

In transport experiments, Caco-2-cells, seeded on 12-well polyester Transwell cell culture inserts (Costar, Cambridge, USA) of 0.4-μm pore size and 12-mm diameter were used. The medium was replaced every second day for 3–4 weeks. The integrity of the Caco-2 monolayers was determined by measuring the transepithelial electrical resistance (TEER) with an epithelial volt-ohmmeter EVOM-G (World Precision Instruments, USA). Only cell monolayers with an initial TEER value above 400 $\Omega\,cm^{-2}$ were used.

On the day of the experiment, the cells were washed and incubated for 20 min in HBSS buffer (pH 7.4). Afterwards, the apical solution was replaced with 910 μL of HBSS containing 0.5% (w/v) of polymers and 1.8% NaFlu. PCP-Cys/GSH (0.5%/0.5%) was used as positive control (Bernkop-Schnürch et al 2004a, b). No polymer was applied to the monolayers, as a negative control. Every hour, 2 volumes of 50 μL were taken from the basolateral solution and replaced with an equal volume of HBSS. The duration of each experiment was 180 min. The concentration of NaFlu in the basolateral solutions was quantified by measuring the fluorescence intensity at an excitation wavelength (λ_{exc}) of 485 nm and emission wavelength (λ_{em}) of 520 nm using a plate reader (Fluostar Galaxy; BMG Labtech, USA). The apparent permeability coefficient (P_{app}) was calculated using equation 4:

$$P_{app} = Q/(A \times c \times t) \quad (4)$$

where P_{app} is the apparent permeability coefficient ($cm\,s^{-1}$), Q is the total amount permeated within the incubation time (μg), A is the diffusion area of the Transwell inserts (1.1304 cm^2), c is the initial concentration of the marker in the donor compartment ($\mu g\,cm^{-3}$) and t is the total time of the experiment (s).

In-vitro evaluation of mucoadhesive properties

Mucoadhesive properties were evaluated by a rotating cylinder method developed in our research group (Bernkop-Schnürch et al 2003). Lyophilized Pec-Cys, Pec or Carbomer 934P (as a positive control) (Fu et al 2002) were compressed with constant pressure into 30-mg, 5.0-mm-diameter flat-faced tablets (single-punch eccentric press; Paul Weber, Germany). The tablets were put on a freshly excised intestinal porcine mucosa, attached to a stainless-steel cylinder by cyanoacrylate glue (diameter 4.4 cm, height 5.1 cm, apparatus 4-cylinder USP). The cylinder was placed in the dissolution apparatus, entirely immersed in 900 mL of PBS at 37°C and agitated at 125 rev min^{-1}. The time required for complete erosion or detachment of the test tablets was determined visually during an observation period of 12 h.

Evaluation of swelling behaviour of the tablets

The tablets produced in the same manner mentioned above were put in USP apparatus I (basket) and entirely immersed in 900 mL of PBS at 37 °C and agitated at 125 rev min^{-1}. The tablets were removed at every time point, blotted on filter paper to remove the excess water, and weighed. During the swelling studies, the weight changes do not always follow an increasing trend. For example, upon erosion of the polymeric matrix, negative changes are also observed. Hence, the term swelling–erosion ratio (SER) was coined and employed throughout this study. The SER was calculated by equation 5:

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

where W_t is the tablet weight at the given time point and W_0 is the initial weight of the tablet. Positive SER and upward trend denote overall swelling and weight gain with water absorption, while negative SER and downward trend demonstrate the erosion. SER of 100% is when the tablets are in the form of a shapeless loose gel or have dissolved completely. In this case, the tablets cannot be handled properly for weighing.

Potential of the pectin–cysteine conjugate as a bead-forming matrix

Preparation of hydrogel beads

The polymers (Pec or Pec-Cys) were hydrated, stirred in insulin solution, and used for bead preparation. Insulin can only be dissolved in acidic aqueous solutions. Twenty milligrams of insulin powder was first dissolved in 8 mL HCl solution at pH 2.0. The pH was slowly increased to 3.99 with NaOH (1 M) and the volume was adjusted to 10 mL with distilled water. This solution was used for hydration of the polymers. The gel solution was employed to prepare the beads by ionotropic gelation. A 5% solution was used in the case of Pec, based on the results from a former study conducted by our group (Atyabi et al 2005). Pec-Cys, on the other hand, formed a highly viscous solution. The highest concentration that could be extruded through the needle was 2.5%. The solution was dropped from a syringe connected to a 20 G flat-tipped injection needle, into a cross-linking solution of zinc acetate (15 mL of cross-linking solution per 3 mL of the gel solution). The beads were cross-linked for a period of 3 h. In the case of oxidized beads, 10 μL of hydrogen peroxide (3%) was also added to the cross-linking solution. Thereafter, the beads were washed three times with distilled water and dried at 10°C, in the dark.

Evaluation of swelling-erosion behaviour of the beads

In swelling–erosion behaviour experiments, pre-weighed amounts of dry beads were evaluated under the same conditions stated below for release studies. The beads were removed at every time point, blotted on filter paper to remove the excess water, and weighed. The swelling–erosion ratio was calculated by equation 5.

Drug release studies

Since insulin is not soluble in common buffers of pH 4-8, sink conditions were not maintained in these buffers and erratic and incomprehensible results were obtained (e.g. in USP phosphate buffer solution pH 7.4 and PBS pH 7.4) (data not shown). Therefore, various buffers were tested and PBS, containing 0.005 mM EDTA and 0.005 M Tris was chosen as the release medium, simulating the intestinal pH. In this buffer insulin was completely soluble to concentrations of 6 mg mL^{-1} and the sink condition was observed (Quinn & Andrade 1983). Moreover, in this study, due to the limit of detection of insulin and limitations in the bead generation and laboratory-scale synthesis of the ingredients, particularly for Pec-Cys, a modified dissolution method developed by Zhang et al (2002) was used for dissolution experiments of multi-particulate dosage formulation.

The release experiments were performed in Eppendorf tubes supported by plastic stands on a waterproof multipoint magnetic stirrer (Variomag, USA) submerged in a water bath at 37°C. Twenty beads were stirred at 100 rev min^{-1} using magnetic stir bars in each Eppendorf tube. Each contained 1 mL of USP acidic buffer medium (KCl/HCl, pH 1.5) and was stirred for 2 h. The medium was replaced with 1 mL of PBS as described above and stirred in the water bath for an additional 6 h. Samples of 250 μL were taken every hour. This same volume was replaced in each tube. Fifty microlitres of 96% ethanol was added to the samples and centrifuged at 13 400 rev min^{-1} for 15 min to precipitate the polymer. The samples were kept at 4°C until further analysis later the same day. Insulin loading in the beads was determined by hydrating and crushing 20 beads in 1 mL of the aforementioned buffer. The analysis of samples for total amount of insulin was performed as described for release study samples.

HPLC analysis of insulin

Insulin concentration in the samples was determined using reversed-phase HPLC-UV at 200 nm, using a modification of the method developed by Dorkoosh et al (2002). The chromatographic system (Merck Hitachi) consisted of Elite LaChrom L-2200 autosampler, L-2130 pump and L-2450 diode array detector. Isocratic elution was performed using 28% acetonitrile and 72% buffer containing 0.1 M KH_2PO_4 and 1% triethyl-amine adjusted to pH 3.0 with phosphoric acid. The column used was LichroCART 75–4 (Merck). The flow-rate and injection volume were 1 mL min^{-1} and 90 μL, respectively. Insulin was detected at a retention time of 2 min, and the detection limit was 3 μg mL^{-1}.

Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance and Tukey post-hoc test. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Characterization of synthesized pectin–cysteine conjugate

Pec-Cys displayed 892.27 ± 68.68 μmol thiol groups per gram of polymer (means ± s.d., n = 3), whereas Pec, on the other hand, was incubated with cysteine in the absence of carbodiimide activating agent EDAC and exhibited no reducing properties. Remaining traces of unbound Cys after dialysis were determined with TNBS reagent to <1% (w/w) of the total mass of the polymer. The lyophilized Pec-Cys appeared as a white and odourless spongy structure. Upon dispersion in distilled water, it formed a semi-transparent gel of high viscosity.

Evaluation of biodegradability

Viscosity measurement

The viscosity of a 1% solution of Pec at 25°C (pH 4) was determined to be 225.8 (± 21.1) mPa s, whereas Pec-Cys and oxidized Pec-Cys showed an increased viscosity of 358.37 (± 25.7) and 1225.35 (± 112.1) mPa s, respectively. The viscosity values demonstrate that thiolation and successive oxidation improves the cohesive properties of Pec-Cys, possibly by formation of inter- and intra-chain di-sulfide bonds.

As illustrated in Figure 2, upon addition of Pectinex Ultra SPL, the viscosity of samples decreased, indicating possible depolymerization by the enzyme. For Pec-Cys and Pec-Cys-ox, the relative reduction in viscosity was more dramatic, probably due to the higher initial viscosity values, compared with Pec. In all cases, the reduction in viscosity seemed to reach a plateau after 10–20 min, indicative of completion of the enzymatic reaction. Viscosities for all of the samples at 30 min post-incubation with Pectinex were in the range of 160–200 mPa s. This might imply that all the samples were broken down by the enzyme to the same extent by the end of the experimental period.

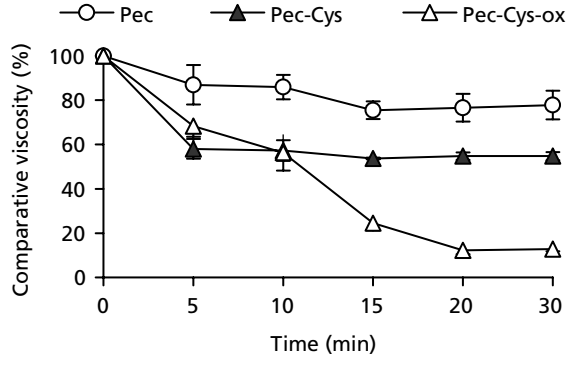

Figure 2 Decrease in viscosity of polymers upon addition of pectinolytic enzyme determined by cone and plate viscometer. Results are expressed as mean ± s.d. of three experiments.

Enzymatic assay by titration method

The main goal of this experiment was to evaluate the biodegradability of thiolated pectin in the presence of pectinolytic enzyme. The reducing property of galacturonic acid, which is produced during enzymatic biodegradation of the polymers, was the basis of this assay. Moreover, since during this experiment it was observed that thiolated pectin (without addition of pectinolytic enzyme) possessed some reducing activity, this observation and its possible explanation was also addressed.

The difference of $Na_2S_2O_3$ volumes used to titrate the excess unreduced I_2 remaining in blank and test samples (V_e) was an indirect indicator of galacturonic acid production and, hence, enzymatic biodegradation of the polymer. V_b and V_t values were inversely proportional to the amount of reducing species (such as galacturonic acid) in the samples. Some points could be deduced from a careful study of Table 1. The V_b value was lower in Pec than Pec-Cys and Pec-Cys-ox. This shows that even without the enzyme being added, Pec-Cys-ox and Pec-Cys have a higher degree of reducing capacity when compared with Pec. This is due to the reducing properties of the thiol groups on the thiolated polymers, which reduce some of the iodine. Furthermore, V_e values in all cases show that addition of enzyme causes production of more reducing species, such as galacturoninc acid, which is an indicator of biodegradation of the polymers. However the V_e values are slightly lower in the case of Pec-Cys and oxidized Pec-Cys, compared with Pec, which might be due to higher viscosity and steric hindrance and the short incubation time (5 min) used in this complementary test, which might have not allowed the completion of the enzymatic activity.

Nevertheless, the results of the viscosity measurement studies, together with the complementary enzymatic assay test, demonstrate that the Pec-Cys and Pec-Cys-ox are degraded by pectinase in-vitro.

Cytotoxicity studies

As shown by the MTT assay, none of the polymers affected the cell viability compared with the negative control (Figure 3A) at time points of either 3 or 16 h. In the LDH test (Figure 3B), Pec-Cys (0.5 and 0.25%) exhibited about 10% toxicity in 3 h, and slightly altered the cell membrane leakage of LDH. Unmodified pectin, which is part of everyday diet and considered a safe polymer, was shown to have 4% toxicity, compared with control. However in 16 h, membrane LDH leakage was almost the same in Pec, Pec-Cys and control cells, and no toxicity was observed. Moreover, by microscopic observation of the cell culture plates, the cells in the control and test wells appeared the same. For example, the cells were attached to the bottom of the wells. This is an indication of their viability. Furthermore, no serious damage to the cells could be seen. The higher initial LDH leakage in the case of Pec-Cys samples might be due to the dramatic change of osmolarity of the culture medium, which is caused by the highly viscous polymer. These data tentatively show that Pec-Cys polymer poses no severe toxicity risk to in-vitro Caco-2 cells at the concentrations and time periods tested.

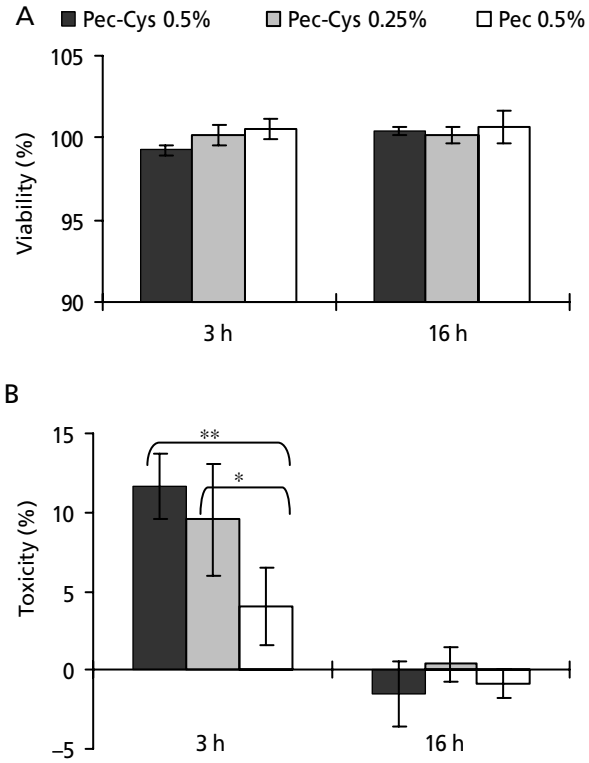

Figure 3 Results of toxicity assays in Caco-2 cell monolayers after incubation times of 3 h and 16 h with polymers. Results are expressed as mean ± s.d. of three experiments. A. Results of MTT assay. B. Results of LDH release test. $*P < 0.05$, $**P < 0.005$.

Table 1 Volumes of $Na_2S_2O_3$ used for titration of excess iodine in enzymatic biodegradation studies

Polymer	V_b[a] (mL)	V_t[b] (mL)	V_e[c] (mL)
Pec	4.95 ± 0.07	4.6 ± 0.0	0.35 ± 0.071
Pec–Cys	3.3 ± 0.0	3.05 ± 0.07	0.25 ± 0.07
Pec–Cys-ox	3.2 ± 0.0	3 ± 0	0.2 ± 0.0

[a]V_e is an indirect indicator of galacturonic acid production and hence biodegradation of the polymer by the enzyme, $V_e = V_b - V_t$; [b]V_b is the volume (mL) to titrate the excess iodine in blank; [c]V_t is the volume (mL) to titrate the excess iodine in test. Results are expressed as mean ± s.d. of three experiments.

Transport experiments

The effect of Pec-Cys on the permeation of NaFlu through Caco-2 cell layers is demonstrated in Figures 4A and 4B. Pec-Cys exhibited a relative enhancement ratio of 1.61 compared with unmodified pectin. On the other hand, Pec-Cys together with permeation mediator reduced glutathione (GSH) showed lower permeation-enhancing effect, compared with Pec-Cys alone. The permeation-enhancing effect of GSH is attributed to inhibition of protein tyrosine phosphatase (PTP) (Bernkop-Schnürch et al 2004a, b). Accordingly, the inhibition of PTP by reduced glutathione will lead to phosphorylation of tyrosine residues of occludin and opening of the tight junctions. Nevertheless, the inhibitory effect

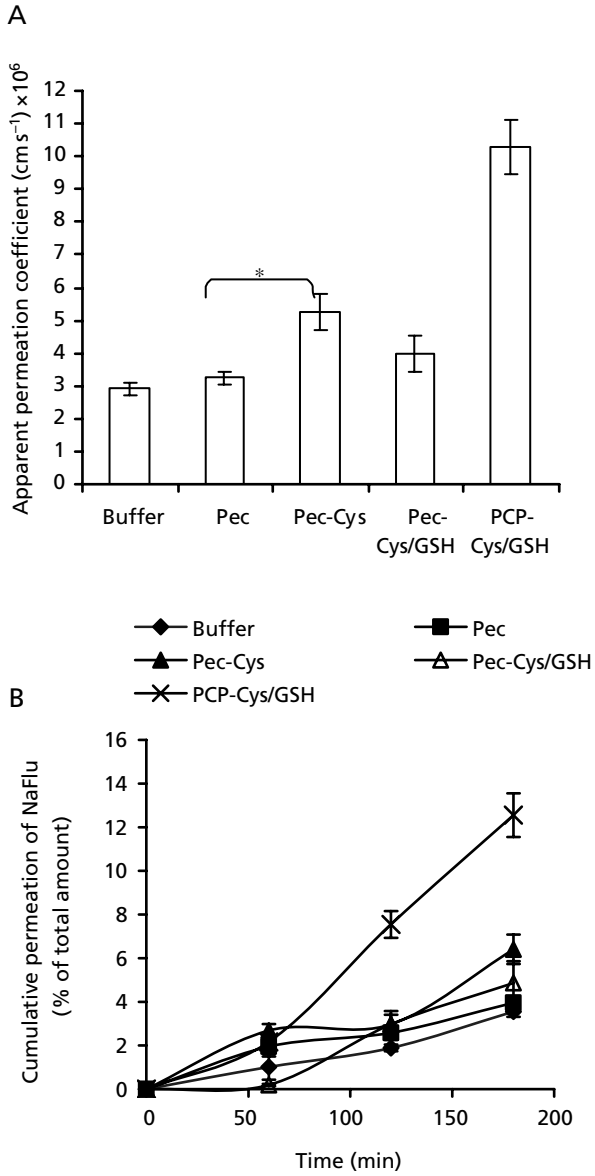

Figure 4 Effect of polymers on permeation of NaFlu through Caco-2 cell monolayers. Results are expressed as mean ± s.d. of three experiments. A. Apparent permeability (P_{app}) for NaFlu transport across Caco-2 cell monolayers. *$P < 0.005$. All groups showed significant difference with PCP-Cys/GSH group ($P < 0.005$). The difference is not shown on the diagram for reasons of clarity. B. Cumulative transport of NaFlu across Caco-2 cell monolayers. Transport data are expressed as percentage of the total dose of NaFlu applied to the luminal side of monolayers.

of glutathione is limited as it is rapidly oxidized on the cell surface (Grafstrom et al 1980). The thiol moieties of thiolated polymers have been formerly shown to reduce oxidized glutathione and therefore the concentration of reduced glutathione on the absorption membrane is increased (Clausen et al 2002). Hence, based on former studies, Pec-Cys/GSH was also assessed in this study for its permeation-enhancing potential. However, the paradoxical observation might be due to the fact that reduced form of thiol groups in both the thiolated polymer and GSH are required to obtain the beneficial effect. In this case, the decrease in the overall permeation-enhancing effect of Pec-Cys/GSH compared with Pec-Cys can be tentatively attributed to formation of disulfide bonds between thiol groups of Pec-Cys and GSH. Nevertheless in-depth studies are needed to elucidate the underlying mechanisms of this observation. Pec-Cys showed a less pronounced permeation-enhancing effect compared with PCP-Cys/GSH used as positive control. Further experiments with other paracellular markers and ex-vivo or in-vivo models should be carried out to verify the feasibility of this polymer as an absorption enhancer.

Swelling–erosion behaviour and mucoadhesive properties of pectin–cysteine tablets

Pec-Cys and C934P tablets both showed very similar favourable adhesion properties in the rotating cylinder method (Figure 5A), whereas Pec tablets detached in less than 3 h from the mucosa. The swelling–erosion behaviour of the tablets is demonstrated in Figure 5B. By simultaneous inspection of the swelling–erosion profile of the tablets and their adhesion duration, it can be deduced that apart from inherent mucoadhesive properties of polymers, their cohesive property is consequential on their residence time on mucosa. It is noteworthy that during the experiment neither Pec-Cys nor C934P tablets detached from the mucosa, but they gradually hydrated and eroded. The short adhesion time of Pec tablets could be, in part, due to poor cohesive properties, since Pec tablets were hydrated and disintegrated rapidly (Figure 5A, B). Consequently, it should be noted that surface charge density and chain flexibility are considered to be prerequisites for bioadhesion; nevertheless, the residence time is also dependent on the dissolution rate of the polymer (Nafee et al 2003).

The controlled swelling–erosion profile of Pec-Cys conjugate, and its favourable adhesion duration on mucosa, renders it a promising polymer in different pharmaceutical applications, such as in vaginal, nasal, buccal, ophthalmic and oral routes of administration.

Potential of the pectin–cysteine conjugate as a bead-forming matrix

Thiolation did not alter the ability of Pec to form beads. Pec-Cys formed spherical beads of uniform size, which shrank to 1 (± 0.1) mm after drying.

Evaluation of swelling–erosion behaviour of beads

In some cases, release data alone can be misleading with regards to preservation of the physical shape of a dosage form. For instance, depending on the drug release mechanisms, the beads may lose their spherical structure and consistency and become a shapeless single-unit gel in the simulated gastrointestinal media but at the same time have kept the major amount of the drug unreleased. On the contrary, the beads might retain their physical shape, but release most of their loaded drug through diffusion. Therefore, apart

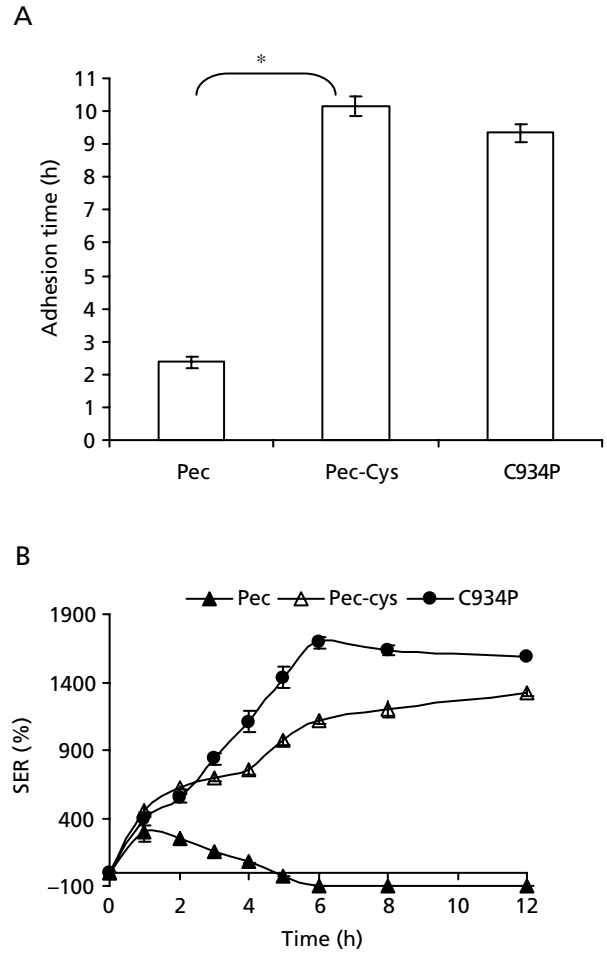

Figure 5 A. Comparison of the adhesion duration of polymeric tablets on the rotating cylinder. Results are expressed as mean ± s.d. of three experiments. *$P < 0.005$. Pec group showed significant difference compared with C934P group ($P < 0.005$). The difference is not shown on the diagram for clarity reasons. B. Swelling–erosion ratio (SER) of polymeric tablets in PBS pH 7.4. Results are expressed as mean ± s.d. of three experiments.

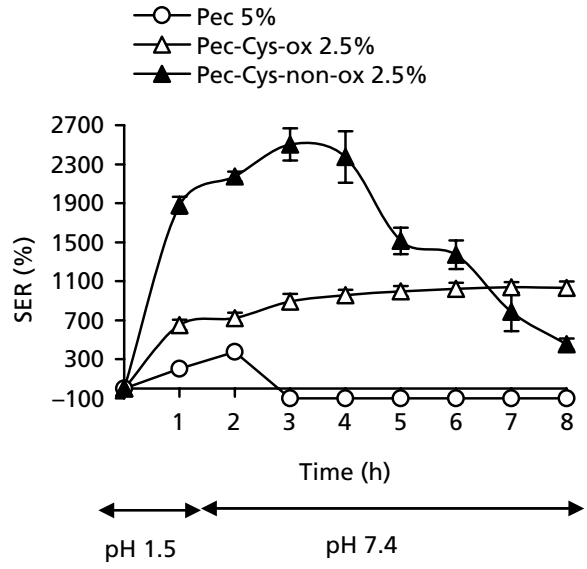

Figure 6 Swelling–erosion ratio (SER) of polymeric beads in acidic buffer pH 1.5 (2 h) followed by PBS (containing Tris and EDTA) pH 7.4 (6 h). Results are expressed as mean ± s.d. of three experiments.

from the release studies, evaluation of swelling behaviour of beads is essential to determine their in-vivo fate and integrity of shape and structure throughout the gastrointestinal tract. On the other hand, the swelling studies elucidate the mechanisms of drug release.

The swelling–erosion profile of polymeric beads is illustrated in Figure 6. In the acidic media, the beads started to gradually swell. Pec beads initially demonstrated a slight swelling, and upon exposure to pH 7.4 buffer were completely eroded within 30 min. The possible explanation of this phenomenon, which has been previously reported (Sibanda et al 2004), is that in the acidic medium the carboxylic group of pectin in the surface of the beads becomes protonated. However, at this pH, due to less hydrophilicity and lack of repulsion between carboxylic groups, cross-linked pectinate (or even uncross-linked pectin) is in the form of a relatively insoluble and tightly packed gel. Thus no significant swelling is observed. At the same time, at this pH, displacement of the cross-linking cation with H^+ or K^+ occurs in the outer layers of beads via diffusion. In this way, the egg-box structure is depleted of its cross-linking agent, but since Pec is insoluble in an acidic environment, the beads keep their spherical structure in macroscopic observation. Thereafter, upon change of medium to a pH of 7.4, the COOH groups once again deprotonate to COO^-, which repel each other. Since some cross-linker is depleted from the beads, the structure is loose and a dramatic swelling and subsequent erosion and drug release occurs.

Non-oxidized Pec-Cys beads showed the most dramatic swelling in this case, with SER approximately 20 fold their initial weight, within 2 h, in acidic medium, followed by gradual erosion upon entering the medium of pH 7.4. This phenomenon, which is not observed in unmodified Pec, is most likely caused by protonation of amine groups of Cys molecules, which causes ionic repulsion in adjacent polymeric chains, and subsequent swelling. The non-oxidized beads had turned into a shapeless gel-like mass in 8 h and further determination of SER was not possible.

Oxidized Pec-Cys beads exhibited a more controlled swelling profile. For example, after initial swelling in the acidic medium, they reached a steady state and kept their spherical shape without marked erosion, even 12 h after being transferred to the pH 7.4 medium. This is most probably due to formation of covalent disulfide bonds within the matrix of oxidized Pec-Cys beads, which stabilized them, even after sequestration of cationic cross-linkers from the matrix. This observation is, in some respects, a breakthrough.

Researchers have studied different aspects of pectinate or alginate beads (Munjeri et al 1997; Sriamornsak & Nunthanid 1998; Pillay & Fassihi 1999a, b; Sriamornsak 1999; El-Gibaly 2002; Atyabi et al 2005). However, in most of these studies the capability of the beads to reach lower gastrointestinal sections as a multiple-unit dosage form is not investigated. In a previous study by our group (Atyabi et al 2005), we observed that

even with utilization of a high concentration of pectin gel solution and zinc acetate as the cross-linker (i.e., instead of calcium chloride gold standard), the beads lose their structure in phosphate buffer after pre-exposure to an acidic environment. The same phenomenon has been reported for alginate beads (Ostberg et al 1994; Aslani & Kennedy 1996). A recent article by Taha et al (2005) also reported the synthesis of thiolated alginate, and studied the behaviour of thiolated alginate beads. They also mention the disintegration of polymeric beads upon exposure to pH 7, after pretreatment in acidic conditions. To date, enteric-coating the beads, or other pectin dosage forms, had been the only practical measure of success to overcome this problem. However, it involves material- and time-consuming stages and is not always practical in laboratory scale. Moreover, by the conventional method of protection of beads in enteric-coated capsules, the advantages of the multiple-unit dosage form are lost. The gastric emptying time is the most important source of variation in the gastrointestinal transit time. The multiple-unit dosage forms act reproducibly and predictively in this regard.

Oxidized Pec-Cys beads could be an alternative solution to this problem. The cross-linking and oxidation process of the beads can take place simultaneously in relatively mild conditions and in a very short time period. Beads with decreased hydrophilicity and controlled swelling are obtained, which can resist the gastrointestinal transit conditions. However, the formulation parameters, such as Pec-Cys gel concentration, degree of cross-linking and oxidation, should be optimized based on the gastrointestinal target area, the physicochemical properties of the therapeutic agent, its mechanism of release and the required release profile.

Insulin release from polymeric beads

The release profile of insulin from the three formulations was nearly identical (Figure 7). Approximately 90% of insulin was released in the first 2 h (pH 1.5). By comparing the release data with swelling erosion profile (Figure 6), it becomes evident that for insulin, the release is predominantly diffusion based. High solubility of insulin in acidic pH plays an important role in this regard. Therefore, even the oxidized Pec-Cys formulation, which showed a favourable stability with respect to physical shape, was not capable of protecting premature insulin release in acidic medium. Consequently, other viable therapeutic candidates should be tested in this regard to further asses the potential of oxidized Pec-Cys beads as a controlled drug delivery system.

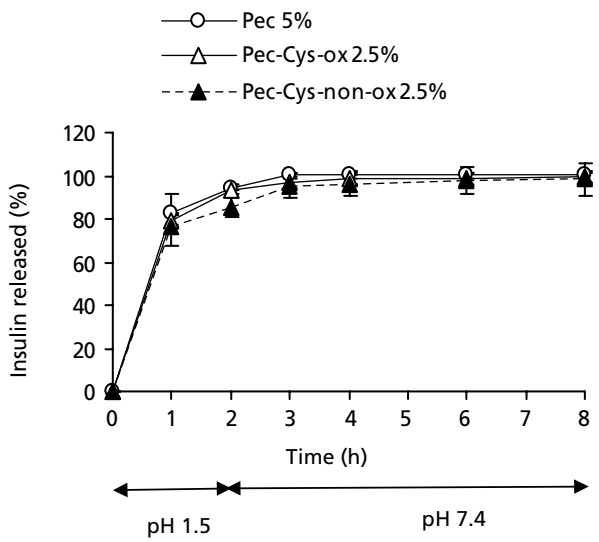

Figure 7 Release profile of insulin from polymeric beads in acidic buffer pH 1.5 (2 h) followed by PBS (containing Tris and EDTA), pH 7.4 (6 h). Results are expressed as mean ± s.d. of three experiments.

Conclusions

In this study, Pec-Cys was shown to have favourable in-vitro biodegradability and low toxicity. The thiolated polymer exhibited a 5-fold increase of adhesion duration, controlled swelling and improved cohesive and permeation-enhancing properties compared with unmodified pectin. Moreover, hydrogel beads of Pec-Cys can be oxidized to dramatically improve their swelling–erosion behaviour and stability in the gastrointestinal tract. On the one hand, the therapeutic agents intended to be loaded for controlled release in these beads have to be carefully chosen based on their physicochemical characteristics and solubility. On the other hand, formulation parameters, such as polymer concentration and cross-linking and oxidation degree, can be optimized to achieve the desired release profiles. Consequently, the new thiolated pectin might be a promising excipient of natural pectin origin, having retained the favourable properties of the pectin backbone, such as biodegradability, low toxicity and ability to form beads. Moreover, unfavourable properties of pectin, such as rapid hydration, swelling and erosion, are no longer present in this new derivative. Pec-Cys conjugate could possibly be used in various transmucosal drug delivery systems intended for vaginal, nasal, buccal, ophthalmic or oral routes of administration. Furthermore, it may show potential for drugs with low solubility.

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