International Journal of Pharmaceutics 404 (2011) 124–132

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

A novel, biodegradable and reversible polyelectrolyte platform for topical-colonic delivery of pentosan polysulphate

Hardik K. Shah[a,*], Jim A. Conkie[b], Robert C. Tait[b], James R. Johnson[a], Clive G. Wilson[a]

[a] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, UK
[b] Department of Haematology, Royal Infirmary, Glasgow G4 0SF, UK

ARTICLE INFO

Article history:
Received 3 August 2010
Received in revised form 1 November 2010
Accepted 10 November 2010
Available online 17 November 2010

Keywords:
Colonic delivery
Polyelectrolyte complex
Chitosan
Poly acrylic acid
Pentosan polysulphate
Bacterial enzymes

ABSTRACT

The goal of the present work was to develop a swellable hydrogel colonic delivery system, which would maximise the availability of the therapeutic agent at a site of inflammation, especially where the water is scarce. A novel method was developed to manufacture a biodegradable and reversible polyelectrolyte complex (PEC) containing chitosan and poly acrylic-acid (PAA). The PEC was analysed using FTIR and DSC, which confirmed the formation of non-permanent swollen gel-network at an alkaline pH. Pentosan polysulphate (PPS) was incorporated in a PEC and an activated partial thromboplastin time assay was developed to measure the release of PPS from PEC. *In vitro* studies suggested that the release of PPS was dependent on the initial drug loading and the composition of the PEC. The gel strength of the swollen network, determined using a texture analyser, was dependent on polymer composition and the amount of PPS incorporated. Bacterial enzymes were collected from the rat caecum and colon for the digestion studies and characterised for glucosidase activity, glucuronidase activity and protein content. The digestion of the reversible polyelectrolyte complexes was measured using a dinitro salicylic acid assay and an increased release of drug was also confirmed in the presence of bacterial enzymes.

1. Introduction

Inflammation is a feature of many diseases and is associated with pain, swelling and tissue remodelling with the formation of non-functional scar tissue. In the gut, the inflammatory process involves the squeezing of pro-inflammatory leucocytes (especially basophils and neutrophils) through the space between endothelial cells (He, 2004). The process leads to the thickening of the bowel lining and disturbs the absorption and motility patterns. The defective endogenous sulphated glycosaminoglycans (GAG) are no longer able to provide a protective barrier following the leucocyte attachment to the gastrointestinal lining.

Yayon et al. (1991) showed exogenous heparin-induced accelerated-healing in mutant Chinese hamster ovary cell-lines suggesting that the presence of low affinity heparin sulphate proteoglycans (HSPGs) was necessary for the mitogenic action of basic-fibroblast growth factor. Papa et al. (2000) noticed an anti-inflammatory action of heparin, which affected the activity of cell adhesion molecules. Day and Forbes (1999) administered heparin to patients with inflammatory bowel disease (IBD) and found the accelerated ulcer healing of the inflamed bowel. Systemic administration of heparin by i.v. and s.c. route caused a significant reduction in stool frequency, C-reactive protein and improved endoscopic and histopathological scores in the IBD patients which strongly suggested its participation during the healing process (Ang et al., 2000). However, the parenteral administration of heparin is always associated with the risk of haemmorhage, suggesting the employment of less active short chain heparin-like molecules.

Törkvist et al. (1999) conducted a clinical trial utilising a low molecular weight heparin for the treatment of glucocorticosteroid-refractory ulcerative colitis and showed improved healing. Heparin and pentosan polysulphate, which has structural motifs similar to heparin, stimulated epithelial proliferation in cultured rat-intestinal epithelial cells (Flint et al., 1994). With regard to pharmacological activity, pentosan polysulphate has one tenth of the anticoagulant activity of heparin. Fischer et al. (1982) studied the inhibitory effects of pentosan polysulphate sodium (PPSNa) and heparin on thrombin, factor Xa and factor IXa in the presence and the absence of antithrombin III. PPSNa was approved as Elmiron® by the U.S. food and drug administration as an oral therapy for the treatment of interstitial cystitis. We investigated the design of a colon-targeted drug delivery platform for PPSNa, intended for the topical treatment of IBD.

Abbreviations: APTT, activated partial thromboplastin time; basic-FGF, basic-fibroblast growth factor; BCA, bicinchoninic acid; CP or Chi:PAA, chitosan:poly acrylic acid; DNSA, 3,5-dinitro-salicylic acid; GAG, glyscosaminoglycans; HSPGs, heparin sulphate proteoglycans; IBD, inflammatory bowel disease; PAA, poly acrylic acid; PEC, poly electrolyte complex; PPS or PPSNa, pentosan polysulphate sodium.

* Corresponding author. Tel.: +353 90 649 5813.
E-mail addresses: hkshah99@yahoo.com, hardik.shah@elan.com (H.K. Shah), c.g.wilson@strath.ac.uk (C.G. Wilson).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.11.012

The delivery of sulphated GAG by the topical route was suggested by Meissner et al. (2007), who entrapped low molecular weight heparin (enoxaparin) into Eudragit® microspheres. Polyelectrolyte complexes composed of chitosan and poly-acrylic acid (PAA), have also been prepared for several biomedical applications. Qu et al. (1997) described the dental applications of a covalently linked chitosan–polyacrylic acid complex. De la Torres et al. (2003, 2005) have prepared amoxicillin-loaded films of chitosan and PAA. The adhesiveness and tensile strength of a transmucosal cast-film system based on a chitosan–PAA complex containing triamcinolone was described by Ahn et al. (2001, 2002). A nanoparticulate ophthalmic drug delivery system, prepared by mixing chitosan solution in acetic acid and a PAA-dispersion in water, has been described for the delivery of pilocarpine (Kao et al., 2006).

The nature of the complex formed between PAA and chitosan described in the literature varies depending on the final usage of the polyelectrolyte complex and encompasses the range between a chemically bound strong-network to an ionically interacted non-permanent assembly. Several authors have described the polymerization of acrylic acid monomers in the presence of chitosan to obtain an inter-penetrated PEC (Lee et al., 1999; Peniche et al., 1999; Ahn et al., 2001; Nge et al., 2004). However, the chemically-linked PAA–chitosan may contain residual toxic acrylic-acid monomers and may display unpredictable swelling behaviour at different pHs (Wang et al., 1997).

Park et al. (2008) manufactured carbopol–chitosan polyelectrolyte complex according to the patent reported in our laboratories (Wilson and Mukherji, 2003). Park et al. (2008) utilised FTIR and DSC to characterise the PEC and confirmed the chemical interaction between two oppositely charged polymers. De Lima et al. (2009) manufactured chitosan–PAA films by allowing diffusion of PAA molecules through chitosan membranes. The authors detected a new covalent linkage at 1560 cm^{-1} during FTIR analysis. These studies suggest that the covalent links between two oppositely charged polymers lead to the formation of a new chemical entity. The complexation of two polymers in solution has lower percentage yield compared to the theoretical yield, which makes it difficult to determine the proportion of individual polymers in the PEC. The unknown amount of individual polymers also makes it difficult to predict the degree of swelling and the mechanical properties of the PEC. The irreversible complex would also have different physico-chemical properties and possibly different physiological effects compared to the individual components.

It is desirable that the oppositely charged polymers in a PEC should not form the covalent bonds in order to utilise the PEC as a drug delivery system. Furthermore, the formulation should swell at neutral to alkaline pH and transport water from the upper gut into the colon to provide water for dissolution. Several hydrogel platforms have been described in the literature as the colonic delivery systems (Simonsen et al., 1995; Pitarresi et al., 2008; Ali and AlArifi, 2009). We utilised chitosan and PAA to form the polyelectrolyte complex for the colonic delivery. The reversible ionic interaction between chitosan and PAA is necessary in order to formulate the biodegradable PEC. The present work addresses these issues and experiments were conducted to formulate a reversible PEC. The bacterial enzymes from rat colon were also utilised to understand the digestion of PEC and its effect on the release of PPS from polyelectrolyte complex.

2. Materials and methods

2.1. Materials

Chitosan (low molecular weight: 50,000–190,000; degree of deacetylation 75–85%, Sigma–Aldrich Co., UK) and poly-acrylic acid (M_v 4,000,000, Sigma–Aldrich Co., UK) were used to manufacture the polyelectrolyte complexes. Other ingredients were sourced as follows: N-acetyl glucosamine (Sigma–Aldrich Co., UK), pentosan polysulphate sodium (bene Arzneimittel GmbH, Germany), potassium dihydrogen orthophosphate (AnalaR, BDH Laboratories, UK), sodium hydroxide (BDH Laboratories, UK), glycine (99%, Sigma–Aldrich Co., UK), 3,5-dinitro salicylic acid (Sigma–Aldrich Co., UK), potassium sodium tartrate (Sigma–Aldrich Co., UK), phenolphthalein (Sigma–Aldrich Co., UK), phenolphthalein-β-D-glucuronide (Sigma–Aldrich Co., UK), p-nitrophenol (Sigma–Aldrich Co., UK), p-nitrophenol-β-D-glucoside (Sigma–Aldrich Co., UK), sodium chloride (BDH Laboratories, UK), EDTA (Sigma–Aldrich Co., UK), bicinchoninic acid kit (Sigma–Aldrich Co., UK), bovine serum albumin (ICN Biomedicals Inc., UK), kaolin/platelet substitute (Diagnostic Reagents Ltd., UK), lyophilised normal human plasma (Diagnostic Reagents Ltd., UK), calcium chloride (Sigma–Aldrich Co., UK).

2.2. Manufacturing of polyelectrolyte complexes (PEC)

Chitosan was dissolved in 0.1 M acetic acid to obtain 1% (w/v) solution. Chitosan was precipitated using acetone, dried overnight and milled to reduce the particle size below 180 μm. Poly-acrylic acid was dispersed in acetone, dried overnight and milled to reduce the particle size below 180 μm. Chitosan (precipitated) and poly-acrylic acid (precipitated) were mixed physically to obtain a PEC containing 1:1 (CP 1:1) and 1:2 (CP 1:2) mass ratios of chitosan and PAA, respectively.

2.3. Characterisation of poly-acrylic acid and chitosan

The samples of poly-acrylic acid and chitosan were characterised by FTIR spectroscopy using a KBr disc method (Genesis Series, FTIR™, ATI Mattson, USA).

The samples of poly-acrylic acid and chitosan were weighed between 3 mg and 5 mg and placed in hermetically sealed pans (Alumina crucible, 40 μL). DSC experiments were performed for PAA and for chitosan between 25 °C and 150 °C at 10 °C min^{-1} using TC 15 (Mettler, Switzerland).

2.4. Activated partial thromboplastin time (APTT) assay for the quantitative determination of PPS

An automatic haemostasis analyser (Amax CS190, *Amelung*®, Germany) was used for the APTT assay. The primary mix of 50 μL of kaolin/platelet substitute and 25 μL of plasma sample were dispensed and incubated automatically at 37 °C for 2 min. Following incubation, calcium chloride (25 μL, 0.025 M) was added automatically to promote the activation of the clotting pathway. Within the measurement cuvette, a small ball-bearing was stopped automatically during the rotation as a result of the clot formation and measuring the clotting time. The assay was controlled using a normal human plasma (clotting time range: 42–48 s) and an abnormal plasma sample (clotting time range: 82–100 s).

A calibration series of pentosan polysulphate (0.01–0.13 mg/mL) was made in phosphate buffer saline. Each calibration standard (100 μL) was aliquoted into 400 μL cups to which normal human pooled plasma (300 μL) was added. The cups were loaded on to the auto sampler and the APTT (sec) was recorded using the Amax CS190. The linear relationship of $\sqrt{\text{Clotting time}\,(s)}$ vs concentration (mg/mL) was plotted and the line of best fit was obtained. Using this reference curve, the activated partial thromboplastin time was measured for PPS containing systems to determine the release rates from PEC.

2.5. Collection of rat colonic bacterial enzymes

The caecal and colonic content from male Sprague–Dawley rats was collected immediately after sacrificing the animals as described by Zhang and Neau (2002). The tissue content was harvested using phosphate buffer saline (pH 7.4, 2–4 °C) and centrifuged (35,000 × *g*) twice to collect the supernatant containing extra-cellular bacterial enzymes. The supernatant was filtered through 0.7 μm glass-microfibre syringe filters (Whatman®) and was dialysed against phosphate buffer saline for 20 h using a SnakeSkin® Pleated dialysis tubing (10 kDa MWCO). The protein content of the bacterial enzymes was determined by bicinchoninic acid (BCA) assay. β-D-Glucosidase and β-D-glucuronidase activities of each preparation were studied using the standard substrates as below. Pooled enzyme preparation from different rats was utilised to study the release of PPS from PEC (CP 1:1) in triplicate. Eighteen individual batches of the enzymes were collected from different rats, which were utilised to study the digestion of chitosan, PEC and also to study the release of PPS from PEC (CP 1:2) following 6 h incubation at 37 °C.

2.6. Bicinchoninic acid (BCA) assay for total protein analysis

Bovine serum albumin was used for the preparation of the calibration series in the concentration ranges 0.01 mg/mL and 1.0 mg/mL. Each calibration standard (25 μL) was mixed with BCA working reagent (200 μL) in 96-well-plate. The enzymatic preparation was diluted 10 times using phosphate buffer saline and 25 μL was mixed with BCA working reagent (200 μL) in the same plate. All the solutions were incubated at 37 °C for 30 min and absorbance was measured at 570 nm using an automatic plate reader (Thermo Labsystems, Multiskan Ascent, USA), which was programmed (Ascent software 2.6) to shake plates for 10 s prior to measurement.

2.7. β-D-Glucosidase and β-D-glucuronidase activity measurements

A calibration series of p-nitrophenol (0.01–0.5 mg/mL) was prepared in distilled water. The calibration standards (50 μL) were pipetted onto 96-well-plate. 20 μL of p-nitrophenol-β-D-glucoside (3.0–4.0 mg/mL in distilled water) and pooled enzyme preparation (30 μL) were mixed in the Eppendorf tubes and incubated for periods between 15 min and 3 h at 37 °C (*n* = 3 per group). 1.0 M NaOH (200 μL) was added after the incubation to denature the proteins and stop the enzyme activity.

Glycine buffer was prepared by dissolving glycine (0.75 g) and NaCl (0.58 g) in distilled water (100 mL). NaOH (0.4 g) was dissolved in this solution and the final volume was made up to 200 mL in a volumetric flask using distilled water. A calibration series of phenolphthalein (0.01–1.0 mg/mL) was made using ethanol–water mixture (50:50, v/v). The calibration standards (50 μL) were pipetted onto a 96-well-plate. 10 μL of phenolphthalein-β-D-glucuronide (4.0–5.0 mg/mL in distilled water), 10 μL of EDTA (0.005 M) and pooled enzyme preparation (30 μL) were mixed in Eppendorf tubes and incubated for periods between 15 min and 3 h at 37 °C (*n* = 3 per group). Glycine buffer (200 μL) was added after the incubation to denature the proteins and to stop the enzyme activity.

Eighteen individual enzyme preparations were analysed for the glucosidase activity in the presence of pentosan polysulphate. The reaction mixture composed of 15 μL of p-nitrophenol-β-D-glucoside (5.0 mg/mL in distilled water), 5 μL of pentosan polysulphate (2.0 mg/mL in distilled water) and enzyme preparation (30 μL). The components were mixed in the Eppendorf tubes and incubated for periods between 15 min and 6 h at 37 °C (*n* = 3 per group). 1.0 M NaOH (200 μL) was added after the incubation to the above solutions.

The absorbance of substrate–enzyme samples and calibration standards were measured simultaneously at 405 nm for β-D-glucosidase activity and 540 nm for β-D-glucuronidase activity using an automatic plate reader (Thermo Labsystems, Multiskan Ascent, USA). The enzyme preparation was mixed with the alkali and followed by the addition of the substrate for the background correction. The instrument was programmed (Ascent software 2.6) to shake plates for 10 s before any measurements. Simultaneous measurements were necessary to avoid any variations in the absorbance readings and to determine the enzymatic activity. The activity was reported as the rate of the substrate breakdown (micromole) in an hour by the amount of protein (mg) present in the enzymes.

2.8. Dinitro salicylic acid assay for the determination of reducing sugars from chitosan and PEC

3,5-Dinitro-salicylic acid (DNSA) reagent was prepared by dissolving sodium hydroxide (0.4 g) and sodium potassium tartrate (7.5 g) in distilled water (10 mL). DNSA (0.025 g) was dissolved and the final solution was made up to 25 mL in a volumetric flask using distilled water (Miller, 1959). A calibration series of N-acetyl glucosamine (0.024–0.24 mg/mL) was prepared in distilled water. Each calibration standard (0.5 mL) was mixed with DNSA reagent (0.5 mL) in a small screw cap vial and boiled in a water bath (100 °C) for 15 min. The solutions were cooled on ice immediately after boiling and absorbance was measured at 500 nm (λ_{max}) using a UV–vis spectrophotometer.

Chitosan (0.05 g, *n* = 6) and PEC (CP 1:1, 0.1 g, *n* = 6) were incubated (37 °C) in the presence of bacterial enzymes (10.0 mL) for 6 h. The solutions were filtered using 0.45 μm nylon syringe filters (Whatman®) after the incubation and reacted with DNSA reagent as mentioned above to determine the degradation products equivalent to N-acetyl glucosamine.

2.9. In vitro dissolution study of PPS from PEC in the presence and the absence of bacterial enzymes

PPS was mixed with PEC (CP 1:1 and CP 1:2) using a mortar and pestle to obtain the drug loading of 10% (w/w) and 20% (w/w). The mixture was filled into hard gelatine capsules (size 0). A dissolution study was performed in phosphate buffer saline (50 mL, 37 °C, pH 7.4) with continuous stirring using a magnetic stirrer. The stainless steel baskets (10 mesh sieve) containing capsules were placed in phosphate buffer saline. All experiments were performed in triplicate. Aliquots (0.5 mL) were sampled up to 16 h and analysed using the APTT assay to determine the percentage release of PPS. The degree of swelling and the texture properties of the swollen gels were determined after the dissolution study.

A dissolution study in the presence of a pooled rat-colonic bacterial enzyme preparation (50 mL, *n* = 3, 37 °C) was also conducted as above for capsules containing PEC (CP 1:1) loaded with PPS at a concentration of 10% (w/w) (PEC–PPS 10). The samples were collected up to 16 h, which were filtered and analysed by APTT assay. The texture properties of the swollen gels were determined after the dissolution study.

In another experiment, the PEC (CP 1:1) was loaded at a concentration of 20% (w/w) (PEC–PPS 20) and was incubated at 37 °C for 6 h in the presence of different batches of the enzyme preparations (10 mL, *n* = 6). The samples were filtered and analysed by APTT to determine the release of PPS.

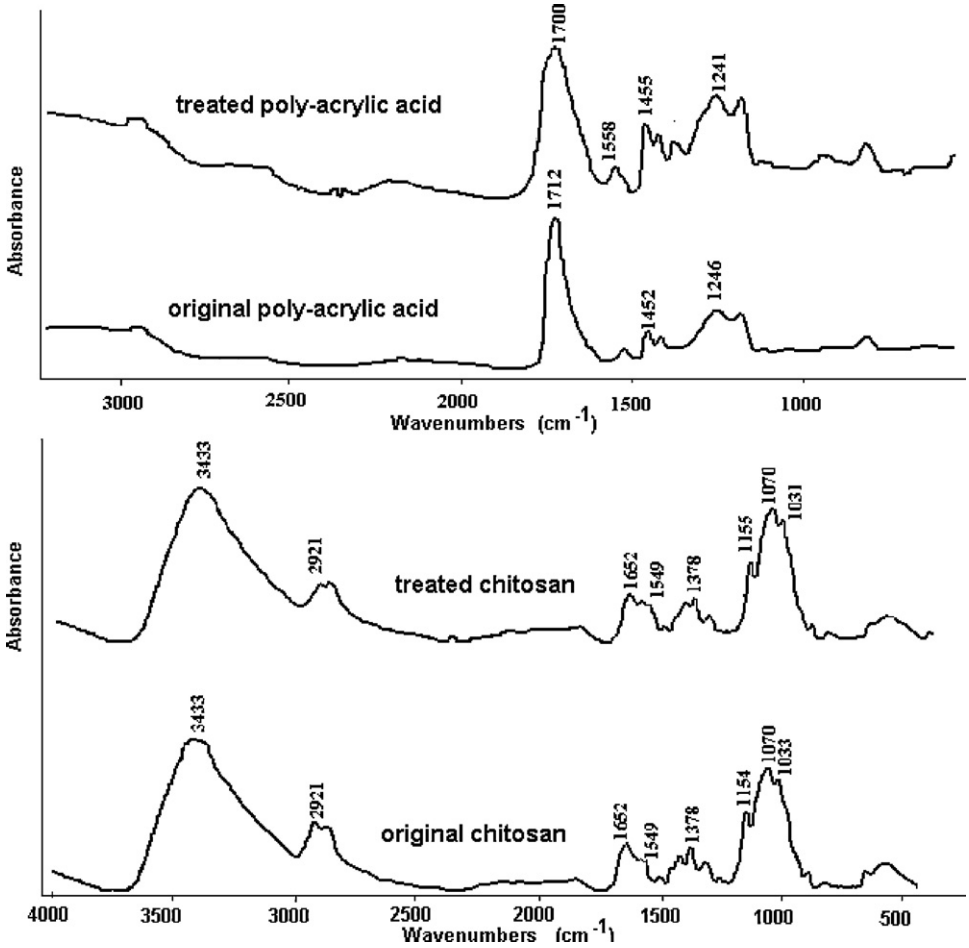

Fig. 1. FTIR plots of chitosan and poly-acrylic acid.

2.10. Binding study of PPSNa and physical mixture

Capsules containing physical mixture (Chitosan:PAA-1:1) were placed in stainless baskets and the baskets were left in phosphate buffer saline (60 mL) containing dissolved PPSNa (2.5 mg/mL). The aliquots (0.5 mL) were collected after 12 h. The experiments were performed in triplicate.

2.11. Determination of the degree of swelling of the swollen PEC

The percentage degree of swelling of PEC was determined by gravimetric analysis after the dissolution studies. The excess surface water was removed by blotting tissue before weighing.

$$\text{Degree of swelling}\,(\%) = \frac{W_h - W_0}{W_0} \times 100$$

where W_h is the weight of PEC after hydration and W_0 is the initial weight of dry PEC.

2.12. Texture analysis of the swollen PEC

The gel strength (firmness) of the swollen PEC (after the dissolution study) was determined using a texture analyser TA-XT2*i* (Stable Microsystems, Surrey, UK) with a 5 kg load cell at room temperature. The swollen gel was placed in a petri-dish, just below the stainless-steel probe (5 mm diameter). The instrument was operated using a Texture Expert Exceed Software (2.51) while setting force in the compression mode. The probe was set to penetrate 3.0 mm through the gel and travel back to the starting point at 1.0 mm/s on detecting the surface of the gel using a trigger force of 0.005 N.

3. Results and discussion

The covalently formed PEC has been proposed as drug delivery system by several authors (Ahn et al., 2002; De la Torres et al., 2003; Kao et al., 2006; Park et al., 2008; De Lima et al., 2009). However, it is difficult to determine the chemical composition of irreversible PEC. If covalently-linked PEC is not fully characterised then it might not be suitable for biomedical applications. The present work focused on the manufacturing of reversibly-linked gel network of polyelectrolyte complex containing two oppositely charged polymers.

A simple physical mixture of originally sourced chitosan and PAA was incubated at an alkaline pH, and it was observed that the mixture did not form a swollen gel network. This control experiment confirmed that original-chitosan remained insoluble and did not interact with original-PAA to form a swollen PEC.

A novel procedure was developed in our laboratory to prepare a fully reversible PEC. Chitosan was dissolved in acetic acid and mixed with an organic solvent to obtain the precipitates of chitosan. PAA was also treated with an organic solvent. The solvent treated chitosan and PAA were characterised using FTIR and DSC. The FTIR plots are illustrated in Fig. 1, which confirmed that chitosan and PAA were not altered chemically after the solvent treatment. DSC plots are illustrated in Fig. 2, which confirmed that initial glass transition temperature (Tgi) of chitosan (130 °C) and PAA (126 °C) was not changed after the solvent treatment. The physical mixture of solvent treated chitosan and PAA (CP 1:1 and CP 1:2) formed a swollen

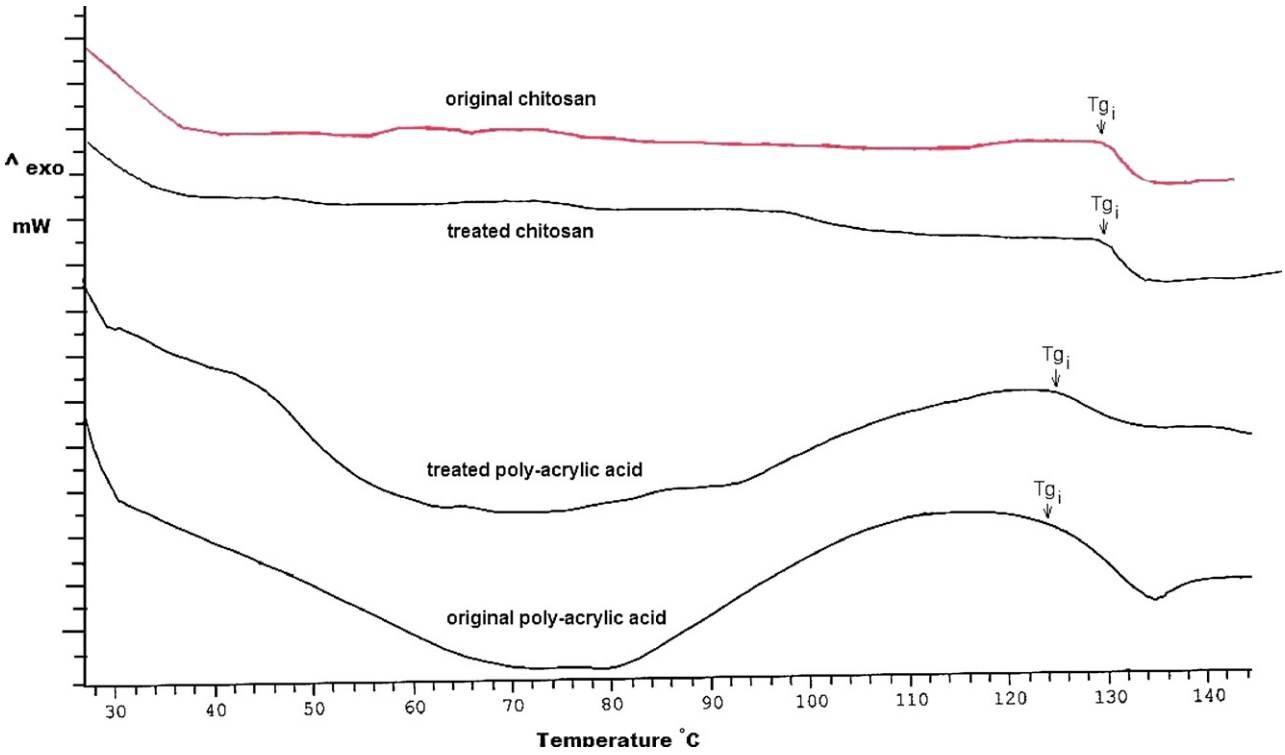

Fig. 2. DSC plots of chitosan and poly-acrylic acid.

insoluble non-permanent gel network at alkaline pH. As chitosan and PAA are not covalently linked in physical mixture, individual components can be completely extracted during acid treatment.

3.1. Analysis of PPS

Quantitative analysis of PPS is difficult as it lacks a chromophore. A chemical assay was attempted for the quantification of PPS after treatment with H_2SO_4. The reaction yielded furfurals giving an absorption peak at 279 nm and the assay allowed the measurement of PPS by UV spectroscopy in the concentration range between 0.02 mg/mL and 0.8 mg/mL (Shah et al., 2005). It was appreciated that the furfural method lacked the necessary sensitivity needed to quantify the release of PPS and as a result PPS was quantified by utilising its anticoagulant property. An assay, based on activated partial thromboplastin times (APTT), was developed to determine the PPS concentrations in the samples. The APTT test was found to be sensitive and linear for the prepared calibration series of PPSNa in phosphate buffer saline. The clotting time of normal human plasma was 42 (±1) s. The concentration of PPSNa from 0.01 mg/mL to 0.13 mg/mL increased the clotting times of normal plasma in the range 49–367 s. The assay allowed the detection of PPSNa between the concentration ranges of 0.0025 mg/mL and 0.025 mg/mL in biological samples. The results confirmed that an APTT assay was 10 times more sensitive compared to the furfural conversion method.

3.2. In vitro release of PPS in the absence of enzymes

Initial dissolution experiments were conducted in our laboratory for 25 h at pH 5.8 and pH 7.6 (900 mL, 37 ± 1 °C) using USP apparatus II (paddle) to understand the release of PPS from PEC (Shah et al., 2005). It was reported that the release profiles of PPS were not different at pH 5.8 and at pH 7.6. The results suggested that the release of PPS would not be affected by the varying pH experienced on the transit through the colon. It is noted that very limited amounts of fluid are available in the colon for the dissolution and diffusion of the active component. Schiller et al. (2005) utilised magnetic resonance imaging to determine the colonic fluid volume for the fed and fasted subjects and estimated that a low free water content was present (13 ± 12 mL). Based on this study, we utilised significantly less amount of dissolution medium (50 mL) as the basis of assessment for the release of PPS.

It was found that the amount of PPS released during the dissolution test was dependent on the composition of the PEC. For example, the PEC (CP 1:2) loaded with 10% (w/w) PPS released a greater amount of PPS compared to the PEC (CP 1:1) (Fig. 3). This suggested that a higher amount of poly-acrylic acid in PEC led to increased release of PPS. Similar differences in release profiles were found for the PEC loaded with 20% (w/w) PPS (Fig. 3). The *in vitro* experiments also revealed that the extent of release of PPS increased with the initial drug loading. It was found that a PEC containing 10% (w/w) of PPS released significantly less amount of PPS after an hour compared to 20% (w/w) PPS loaded PEC ($p < 0.05$, ANOVA, Fig. 3). It was also observed that the complete amount of PPS was not released from PEC after 16 h. This was investigated in further experiments while conducting PPS binding study with poly-electrolyte complex as described in later sections.

The texture properties and the degree of swelling were determined for the PEC loaded with different amount of PPS after completion of dissolution studies (Fig. 4A and B). As shown in Fig. 4A, the gel strength of the PEC was significantly decreased when the proportion of PAA was increased ($p < 0.05$, ANOVA). This confirmed that higher proportion of PAA led to loosely bound gel network with a tendency to absorb a larger amount of water. The gel strength of the swollen PEC (CP 1:1) also decreased significantly with increasing the initial loading of PPS ($p < 0.05$, ANOVA; Fig. 4A). The results suggested that the sulphated polyionic species interfere during the interaction between chitosan and PAA at the simulated colonic pH.

One of the most important functions of the colon is to reabsorb water, which leaves very small amounts of luminal fluid for the distribution of drug beyond the ascending colon. The carrier system

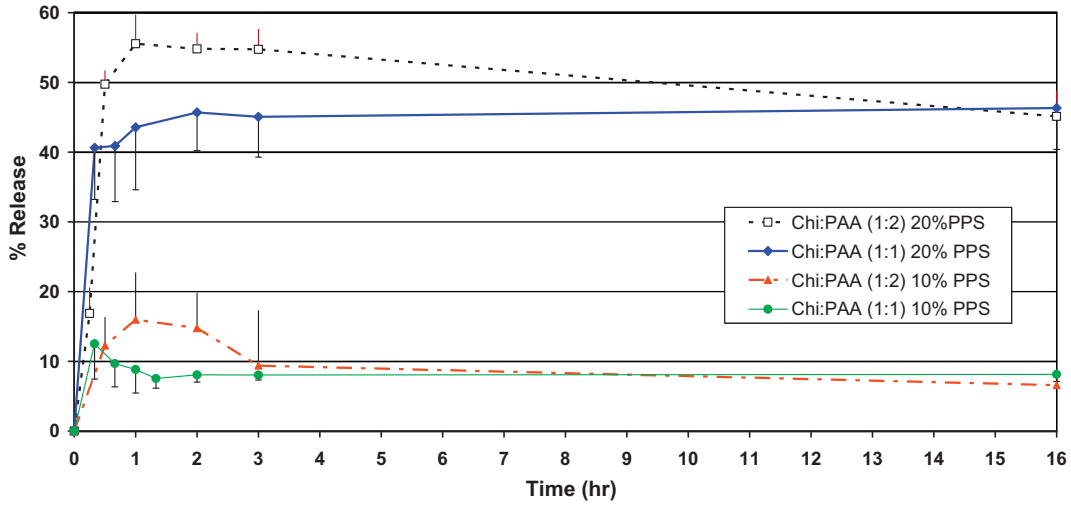

Fig. 3. *In vitro* release of PPS (10% and 20% (w/w) initial loading) from PEC (mean ± SD, $n = 3$).

described here swells at colonic pH absorbing the fluid to more than twenty five times of the original weight (Fig. 4B). We propose that the absorbed fluid assists in the distribution of the incorporated drug and the swollen gel network was able to release a proportion of the entrapped drug. The dissolution studies confirmed that the release characteristics of PPS from the swollen network were dependent on the composition of the polyelectrolyte complex.

3.3. *In vitro release of PPS in the presence of enzymes*

In the design of a colonic delivery system, the anaerobic environment of a colon generated by bacterial flora needs to be considered. The enzymes secreted by these bacteria include β-glucuronidase, β-glucosidase, β-galactosidase, nitro-reductase, azo-reductase, cholesterol dehydrogenase, oligosaccharidases, and polysaccharidases which are responsible for the digestion of soluble complex carbohydrates. There is some argument about whether chitosan is digestible; in the food industry chitosan is regarded as a non-digestible fibre but partially N-acetylated derivatives are degraded by lysozyme and microbial chitinases (Hirano et al., 1989). Vernazza et al. (2005) described a three stage fermentation model of the human colon which was used to investigate the metabolism of chitosan in the presence of various bacterial species including clostridia, lactobacilli and bacteroides and confirmed digestion. McConnell et al. (2008) studied the degradability of chitosan by the human colonic microflora. The authors incubated chitosan films in human faecal slurry and found significant weight loss after 18 h. On the basis of these observations, it was concluded that chitosan was partially digestible (McConnell et al., 2008). The *in vitro* degradation of chitosan has also been studied using the colonic content from the rat colon (Zhang and Neau, 2002), which was selected as a model because the rat microfloral content has been reported to resemble that of humans (Hawksworth et al., 1971). In order to investigate the possible digestion of the PEC, an enzyme preparation was prepared by collection of rat caecal and colonic content based on the method of Zhang and Neau (2002).

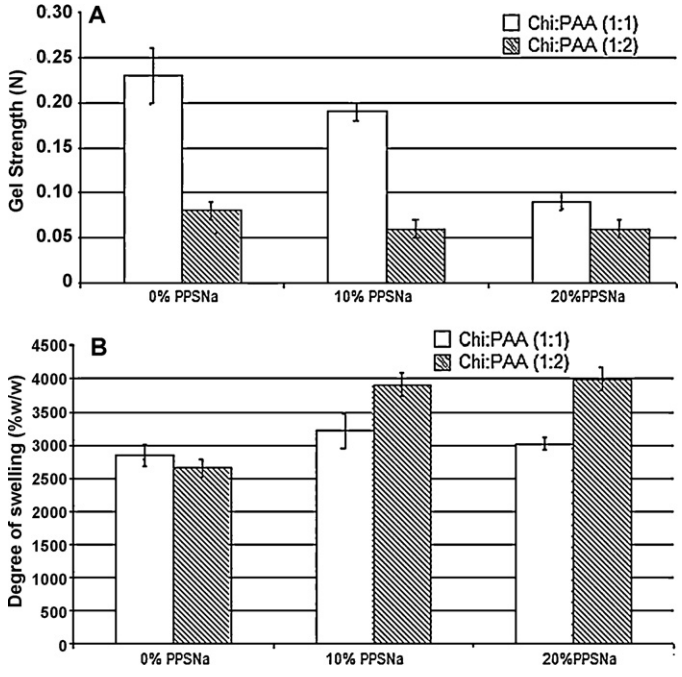

Fig. 4. (A) Gel strength and (B) degree of swelling of PPS loaded PEC (Chitosan:PAA) after dissolution studies at pH 7.4 (mean ± SD, $n = 3$).

In the first experiment, caecal and colonic contents were pooled from several batches of rats in order to obtain sufficient bacterial enzymes for the incubation. The total protein concentration of the colonic enzyme preparation was determined using bicinchoninic acid (BCA) assay, which is based on the Lowry method (1951). The total protein content of the different rat bacterial enzyme preparations varied between 1000 μg/mL and 5000 μg/mL, although the colonic content collected from the rats was approximately standardised (8.3 ± 0.6 g per rat colon, $n = 6$).

The presence of β-D-glucuronidase and β-D-glucosidase was determined by digestion of p-nitrophenol β-D-glucoside and phenolphthalein β-D-glucuronide, respectively according to the methods described by Freeman (1986). However, the final volume of the reaction mixture in the reported assay was about 5.0 mL, which limited the sensitivity of the assay while dealing with very small amounts of the enzyme preparation (Freeman, 1986). The assays were modified to utilise a final volume of a reaction mixture of 250 μL. The assays were conducted in 96-well-plate and the absorbance of all the samples was measured using an automatic plate reader. The formation of digestion products (p-nitrophenol and phenolphthalein) allowed the quantitative determination of the digestion products to determine the activity of the different enzyme preparations (Fishman and Stahl, 1984; Freeman, 1986). Over all, 96-well-plate method was fast, accurate and sensitive. Enzyme preparations digested the substrates in the presence of PPS

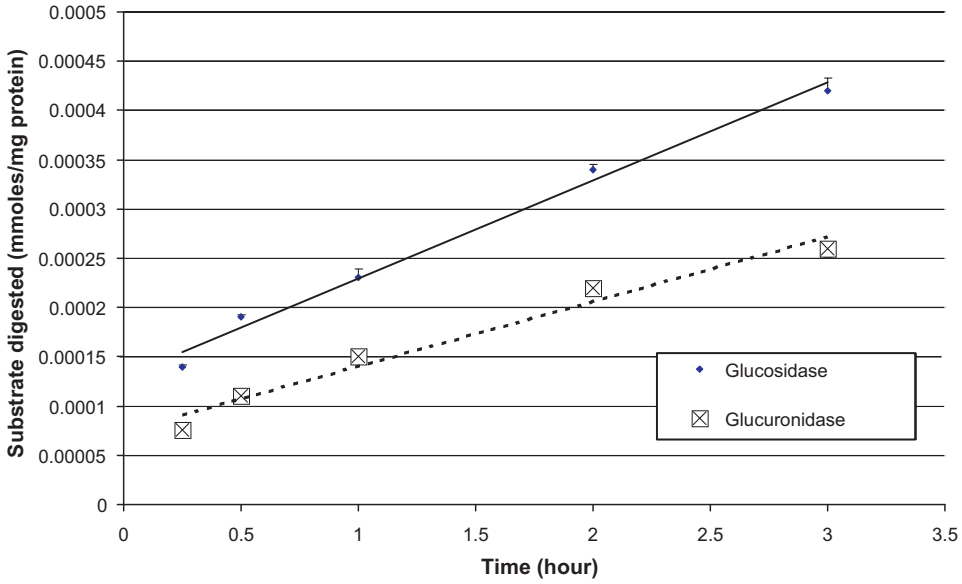

Fig. 5. Breakdown of p-nitrophenol β-D-glucoside and phenolphthalein β-D-glucuronide in the presence of the bacterial enzyme preparation (37 °C, mean ± SD, $n = 3$).

and data suggested that PPS does not interfere with the enzymatic activity on incubation.

As illustrated in Fig. 5, the pooled enzymatic preparation (0.057 mg protein) metabolised the substrates p-nitrophenol β-D-glucoside (0.23 μmol) and phenolphthalein β-D-glucuronide (0.15 μmol) in an hour, which confirmed the presence of glucosidases and glucuronidases, respectively. Eighteen different batches of enzyme preparations were collected from eighteen different rats and their glucosidase activity was confirmed as illustrated in Fig. 6. These enzyme preparations were utilised to study the digestion of chitosan, the digestion of chitosan in the PEC and the release of PPS from PEC.

The degradation of chitosan yields monomers including glucosamine, N-acetyl glucosamine and several oligosaccharides. A dinitro salicyclic acid (DNSA) assay, based on the method described by Miller (1959), was used for the determination of the reducing sugars. Chitosan present in a swollen PEC (0.1 g, CP 1:1) was digested by the enzymes (glucosidase activity: 16.4 ± 6.9 ((μmol/h)/mg), which produced 1.3 mg (standard deviation: 0.30) of the monomeric species equivalent to N-acetyl glucosamine. However, the digestion of chitosan alone by the enzymes (glucosidase activity: 17.4 ± 7.1 ((μmol/h)/mg) produced 0.1 mg (standard deviation: 0.16) of the monomeric species equivalent to N-acetyl glucosamine. The results suggested that chitosan present in PEC was more susceptible to the degradation while forming the electrostatic interaction with PAA in a swollen state compared to chitosan only.

The crude colonic content isolated in this procedure was found not to affect the quantitative determination of PPS by the APTT assay. As a result, it was possible to use this sensitive procedure to measure the effect of bacterial enzymes on the release of PPS from the PEC.

The dissolution study of the PEC (CP 1:1) loaded with PPS at a concentration of 10% (w/w) showed that more amount of drug was released within first 2 h in the presence of enzyme preparation compared to the study conducted in the absence of enzymes (Fig. 7). A decreased amount of drug was found in the dissolution media after initial swelling. This suggested that the swollen gel had a greater affinity for PPS than the non-swollen material.

The texture analysis of the swollen PEC, conducted after the dissolution study, showed that the gel strength of a swollen network was reduced in the presence of the enzymes (0.15 ± 0.01 N) com-

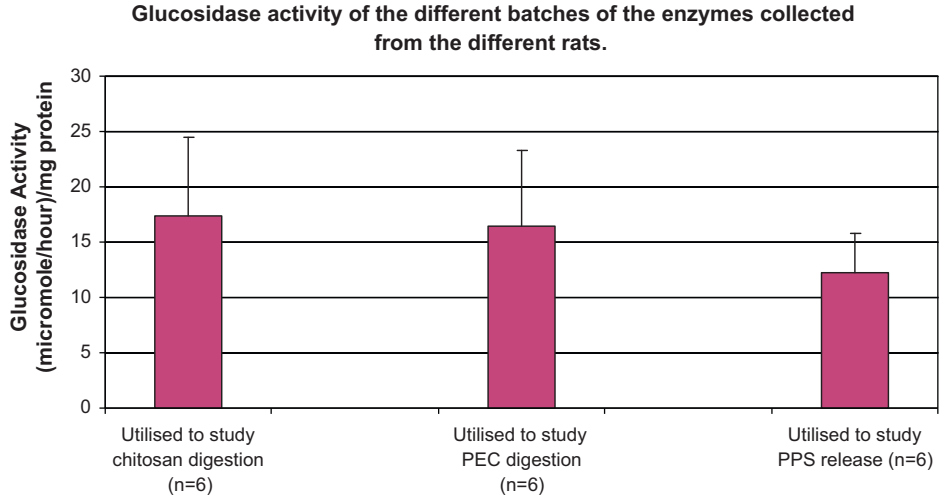

Fig. 6. Glucosidase activity of the different batches of the enzymes utilised for the digestion of chitosan, PEC and also to study the PPS release (mean ± SD, $n = 6$).

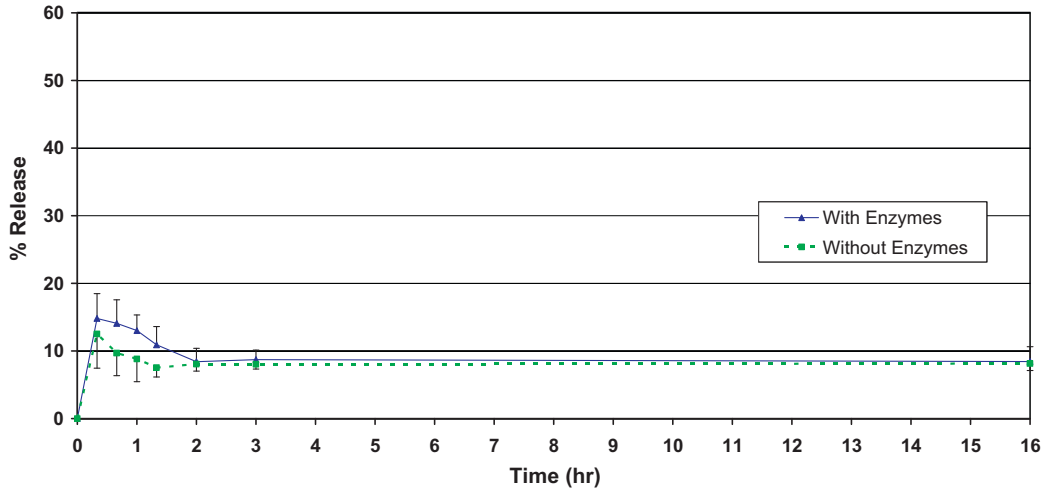

Fig. 7. *In vitro* release of PPS from PEC (Chi:PAA-1:1, 10% (w/w) PPS loading) in the presence and absence of bacterial enzyme preparation (mean ± SD, $n = 3$).

pared to the gel strength in the absence of enzymes (0.19 ± 0.01 N). The results suggested that the PEC was susceptible to the biodegradation in the presence of enzyme preparation.

The enzymatic preparation collected from different rats ($n = 6$) were utilised to incubate the PEC (CP 1:1) containing PPS (20% (w/w) loading) for 6 h. The formulation released 9.2% (±2.6%) of PPS in the presence of enzyme preparation compared to 6.9% (±2.2%) of PPS in the absence of enzyme preparation (i.e. phosphate buffer saline). This study further confirmed the biodegradation of PEC by the bacterial enzymes leading to higher release of PPS.

3.4. Binding study of PPSNa and physical mixture

Swelling of the PEC reached equilibrium within an hour of exposure to buffer and resulted in the maximum release of the PPS during dissolution experiments. The amount of free drug in solution decreased after an hour especially from PEC (CP 1:1). An interpretation is that negatively charged polymeric drug was binding back to the positively charged surface groups (i.e. R-NH_3^+) of the swollen PEC leading to the decreased amount of PPS in solution after swelling. A control experiment was conducted to confirm these electrostatic interactions while incubating the placebo physical mixture (Chitosan:PAA, 1:1) in PPSNa stock-solution (2.5 mg/mL).

APTT studies confirmed that 27.8 ± 3.7% ($n = 3$) of PPSNa was bound to the physical mixture during the control experiment. The results supported the observations made in dissolution studies, during which the released amount of PPSNa was decreased after an hour. A swollen PEC was retained in the fixed volume of dissolution medium during the *in vitro* dissolution experiments. This allowed binding of soluble PPSNa back to the charged surface groups of an insoluble gel network as it hydrated. However, this may be a technical artefact as the drug-loaded PEC will not be retained in a fixed aqueous volume after oral administration and there will be a limited possibility of the electrostatic interaction.

4. Conclusions

A novel method was developed to manufacture the polyelectrolyte complexes (PEC) containing chitosan and PAA, which produced a swollen, insoluble and non-permanent gel network at simulated colonic pH. Texture analysis of the swollen gels confirmed that the composition of PEC affected the cohesion of the gel. As expected, a swollen gel network also became weakened by the incorporation of pentosan polysulphate (PPS). The present work confirmed that the PEC was degradable in the presence of a crude bacterial-enzyme preparation collected from rat colonic and caecal contents. This led to a further release of pentosan polysulphate for the topical action. Chitosan would be charged and solubilised at the gastric pH, as a result the PEC would dissociate in the gastric environment. For this reason, it would be necessary to provide an enteric coating either for PEC–PPS filled capsules or for PEC–PPS tablets. The swollen PEC provides a delivery system containing water, which will aid local diffusion of the drug in the dry environment of the colon and will facilitate the distribution of the drug through the luminal contents to the inflammatory sites of colon wall.

Acknowledgement

We are grateful to bene Arzneimittel GmbH (Germany) for the kind donation of pentosan polysulphate.

References

Ahn, J.S., Choi, H.K., Cho, C.S., 2001. A novel mucoadhesive polymer prepared by template polymerization of acrylic acid in the presence of chitosan. Biomaterials 22, 923–928.
Ahn, J.S., Choi, H.K., Chun, M.K., Ryu, J.M., Jung, J.H., Kim, Y.U., Cho, C.S., 2002. Release of triamcinolone acetonide from mucoadhesive polymer composed of chitosan and poly(acrylic acid) *in vitro*. Biomaterials 23, 1411–1416.
Ali, A.E., AlArifi, A., 2009. Characterisation and *in vitro* evaluation of starch based hydrogels as carriers for colon specific drug delivery systems. Carbohydr. Polym. 78, 725–730.
Ang, Y.S., Mahmud, N., White, B., Byrne, M., Kelly, A., Lawler, M., McDonald, G.S.A., Smith, O.P., Keeling, P.W.N., 2000. Randomised comparison of unfractioned heparin with corticosteroid in severe active inflammatory bowel disease. Aliment Pharmacol. Ther. 14, 1015–1022.
Day, R., Forbes, A., 1999. Heparin, cell adhesion and pathogenesis of inflammatory bowel disease. Lancet 354, 62–65.
De la Torres, P.M., Enobakhare, Y., Torrado, G., Torrado, S., 2003. Release of amoxicillin from polyionic complexes of chitosan and poly(acrylic acid). Study of polymer/polymer complex and polymer/drug interactions within the network structure. Biomaterials 24, 1499–1506.
De la Torres, P.M., Torrado, G., Torrado, S., 2005. Poly(acrylic acid) chitosan interpolymer complexes for stomach controlled antibiotic delivery. J. Biomed. Mater. Res. B 72, 191–197.
De Lima, M.S.P., Freire, M.S., Fonseca, J.L.C., Pereira, M.R., 2009. Chitosan membranes modified by contact with poly(acrylic acid). Carbohydr. Res. 344, 1709–1715.
Fischer, A.M., Barrowcliffe, T.W., Thomas, D.P., 1982. A comparison of pentosan polysulphate (SP54) and heparin I: mechanism of action on blood coagulation. Thromb. Haemost. 47, 104–108.
Fishman, W.H., Stahl, P.D., 1984. In: Bergmeyer, H.U., Bergemeyer, J., Grassl, M. (Eds.), Glycosidases: β-D-glururonidase. Methods of Enzymatic Analysis, vol. 4. Verlag Chemie, pp. 246–255.
Flint, N., Cove, F.L., Evans, G.S., 1994. Heparin stimulates the proliferation of intestinal epithelial cells in primary culture. J. Cell Sci. 107, 401–411.

Freeman, H.J., 1986. Effects of differing purified cellulose, pectin and hemicellulose fiber diets on fecal enzymes in 1,2-dimethylhydrazine-induced rat colon carcinogenesis. Cancer Res. 46, 5529–5532.
Hawksworth, G., Drasar, B.S., Hill, M.J., 1971. Intestinal bacteria and the hydrolysis of glycosidic bonds. J. Med. Microbiol. 4, 451–459.
He, S.H., 2004. Key role of mast cells and their major secretory products in inflammatory bowel disease. World J. Gastroenterol. 10, 309–318.
Hirano, S., Tsuchida, H., Nagao, N., 1989. N-acetylation in chitosan and the rate of its enzymic hydrolysis. Biomaterials 10, 574–576.
Kao, H.J., Lin, H.R., Lo, Y.L., Yu, S.-P., 2006. Characterisation of pilocarpine-loaded chitosan/carbopol nanoparticles. J. Pharm. Pharmacol. 58, 179–186.
Lee, J.W., Kim, Y.S., Kim, S.S., Lee, Y.M., Lee, K.H., 1999. Synthesis and characteristics of interpenetrating polymer network hydrogel composed of chitosan and poly(acrylic acid). J. Appl. Polym. Sci. 73, 113–120.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin-phenol reagents. J. Biol. Chem. 193, 265–275.
McConnell, E.L., Murdan, S., Basit, A.W., 2008. An investigation into the digestion of chitosan (non-crosslinked and crosslinked) by human colonic bacteria. J. Pharm. Sci. 97, 3820–3829.
Meissner, Y., Ubrich, N., Ghazouani, F., Maincent, P., Lamprecht, A., 2007. Low molecular weight heparin loaded pH-sensitive microparticles. Int. J. Pharm. 335, 147–153.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
Nge, T.T., Hori, N., Takemura, A., Ono, H., 2004. Swelling behavior of chitosan/poly(acrylic acid) complex. J. Appl. Polym. Sci. 92, 2930–2940.
Papa, A., Danese, S., Gasbarrini, A., Gasbarrini, G., 2000. Review article: potential therapeutic applications and mechanisms of action of heparin in inflammatory bowel disease. Aliment Pharmacol. Ther. 14, 1403–1409.
Park, S.H., Chun, M.K., Choi, H.K., 2008. Preparation of an extended-release matrix tablet using chitosan/carbopol interpolymer complex. Int. J. Pharm. 347, 39–44.
Peniche, C., Argüelles-Monal, W., Davidenko, N., Sastre, R., Gallardo, A., Roman, J.S., 1999. Self-curing membranes of chitosan/PAA IPNs obtained by radical polymerization: preparation, characterisation and interpolymer complexation. Biomaterials 20, 1869–1878.
Pitarresi, G., Tripodo, G., Calabrese, R., Craparo, E.F., Licciardi, M., Giammona, G., 2008. Hydrogels for potential colon drug release by thiol-ene conjugate addition of a new inulin derivative. Macromol. Biosci. 8, 891–902.
Qu, X., Wrzyszczynski, A., Pielichowski, K., Pielichowski, J., Adamczak, E., Morge, S., Rabek, J.F., 1997. Polymerization of chitosan–acrylic salt for use in dentistry. J. Macromol. Sci. A 34, 881–899.
Schiller, C., Fröhlich, C.P., Giessmann, T., Siegmund, W., Mönnikes, H., Hosten, N., Weitschies, W., 2005. Intestinal fluid volumes and transit of dosage forms as assessed by magnetic resonance imaging. Aliment Pharmacol. Ther. 22, 971–979.
Shah, H., Johnson, J., Wilson, C., 2005. Delivery of poly-ionic pharmacons to the colon using polyelectrolyte complexes. AAPS J. 7, R6130.
Simonsen, L., Hovgaard, L., Mortensen, P.B., BrØnsted, H., 1995. Dextran hydrogels for colon-specific drug delivery. V. Degradation in human intestinal incubation models. Eur. J. Pharm. Sci. 3, 329–337.
Törkvist, L., Thorlacius, H., Sjöqvist, U., Bohman, L., Lapidus, A., Flood, L., Agren, B., Raud, J., Löfberg, R., 1999. Low molecular weight heparin as adjuvant therapy in active ulcerative colitis. Aliment Pharmacol. Ther. 13, 1323–1328.
Vernazza, C.L., Gibson, G.R., Rastall, R.A., 2005. *In vitro* fermentation of chitosan derivatives by mixed cultures of human faecal bacteria. Carbohydr. Polym. 60, 539–545.
Wang, H., Li, W., Wang, Z., 1997. Studies on chitosan and poly(acrylic acid) interpolymer complex. I. Preparation, structure, pH-sensitivity, and salt sensitivity of complex-forming poly(acrylic acid): chitosan semi-interpenetrating polymer network. J. Appl. Polym. Sci. 65, 1445–1450.
Wilson, C.G., Mukherji, G., 2003. Glucosamine–polyacrylate inter-polymer complex and applications thereof. Patent No WO 03/067952.
Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., Ornitz, D.M., 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64, 841–848.
Zhang, H., Neau, S.H., 2002. *In vitro* degradation of chitosan by bacterial enzymes from rat cecal and colonic contents. Biomaterials 23, 2761–2766.