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Synthesis of zinc-crosslinked thiolated alginic acid beads and their *in vitro* evaluation as potential enteric delivery system with folic acid as model drug

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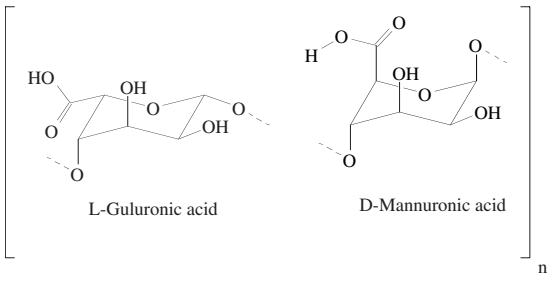
The aim of this study is to explore the potential of synthetic modifications of alginic acid as a method to enhance the stability of its complexes with divalent cations under physiological conditions. A fraction of algin's carboxylic acid moieties was substituted with thiol groups to different substitution degrees through conjugating alginate to cysteine to produce alginate-cysteine (AC) conjugates. Infrared spectrophotometry and iodometry were used to characterize the resulting polymeric conjugates in terms of structure and degree of substitution. Moreover, zinc ions were used to crosslink the resulting AC polymers. Folic acid loaded beads were prepared from Zinc-crosslinked AC polymers (AC-Zn) of different cysteine substitution degrees. The generated beads were then investigated *in vitro* for their capacity to modify folic acid release. AC-Zn polymeric beads resisted drug release under acidic conditions (pH 1.0). However, upon transfer to a phosphate buffer solution (pH 7.0) they released most of their contents almost immediately. This change in drug release behavior is most probably due to the sequestering of zinc cations by phosphate ions within the buffer solution to form insoluble chelates and, to a lesser extent, the ionization of the carboxylic acid and thiol moieties. Removal of zinc ions from the polymeric matrix seems to promote polymeric disintegration and subsequent drug release. A similar behavior is expected *in vivo* due to the presence of natural zinc sequestering agents in the intestinal fluids. AC-Zn polymers provided a novel approach for enteric drug delivery as drug release from these matrices complied with the USP specifications for enteric dosage forms.

1. Introduction

Sodium alginate (algin) is the purified carbohydrate product isolated from brown seaweads. Algin consists chiefly of the sodium salt of alginic acid, which is a linear copolymer of 1,4-linked mannopyranosyluronic acid and 1,4-linked gulopyranosyluronic acid units. Alginic acid has received great interest as drug delivery matrix prompting the publication of recent reviews dealing with this area (Shilpa et al. 2003; Raj and Sharma 2003).

Alginate has been investigated as a carrier material in different controlled release systems (Pillay and Fassihi 1999a, 1999b). It was employed in the preparation of controlled release microspheres or minimatrices for a variety of medicinal agents including metoclopramide and cisapride (Al-Musa et al. 1999), diclofenac (Fernandez-Hervas et al. 1998), indomethacin (Pillay et al. 1998; Shiraishi et al. 1993), propranolol (Lim and Wan 1997), gentamicin (Iannuccelli et al. 1996) and others. Furthermore, alginic acid was recently used to encapsulate chitosan bioadhesive microspheres, and *vice versa*, for intestinal drug delivery (Ramdas et al. 1999; Gaserod et al. 1998). Recently, alginate was developed as a nanoparticle for the oral delivery of insulin (Raj and Sharma 2003).



Algin is also characterized with useful gel-forming properties when mixed with different polyvalent cations (Takka and Acarturk 1999a; Aslani and Kennedy 1996). Calcium alginate has found applications in a number of gelation purposes including the formation of a firm gel for the preparation dental impressions (Steas 1991), and in the preparation of matrices for drug delivery (Kamath and Park 1993).

The ratio of mannuronic acid to guluronic acid strongly influences the drug releasing properties of calcium alginate beads (Shiraishi et al. 1993; Takka and Acarturk 1999a; Acarturk and Takka, 1999b). A recent study has indicated that polymeric beads of calcium-alginate incorporating guluronic acid block (an alginate hydrolyzates)

and chitosan yielded effective controlled release of hydrocortisone (Murata et al. 2004). Chitosan-alginate-electrolyte matrices were found to be superior to their chitosan-carrageenan counterparts in providing prolonged drug release profiles (Tapia et al. 2004).

However, the drug releasing properties of calcium alginate matrices were reported to suffer from some problems. Their dissolution in phosphate buffered saline solution (pH 7.4) occurs completely within a short period after a certain lag time (Aslani and Kennedy 1996; Kikuchi et al. 1997; Ostberg et al. 1994). Additionally, the matrices were able to extend the release of theophylline and chloramphenicol only when pure water was applied as release medium. While in 0.1 M HCl, simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and 0.1 M NaCl the drug release proceeded much more rapidly. The crosslinking calcium ions were rapidly discharged from the matrices in the presence of acid to yield the protonated alginic acid. This transformation reduced the degree of crosslinking within the matrix, and thus destroyed its ability to provide retarded drug release. In NaCl solutions and SIF, calcium ions were partly exchanged by the non-gelling sodium ions or sequestered by phosphate. This caused swelling and, in the latter case, dissolution of the matrices, thus inducing rapid release of the encapsulated drug. Accordingly, it was concluded that calcium alginate minimatrices do not seem applicable as an oral controlled release system, due to the pronounced sensitivity towards the composition of the release medium and the rapid drug release in media of physiological relevance (Ostberg et al. 1994).

These problems prompted our quest for suitable synthetic modifications of alginic acid to enhance the stability of its corresponding complexes with divalent cations under physiological conditions. We focused our attention on conjugating alginic acid to organic moieties characterized by a high affinity to divalent cations. For example, conjugating hydroxamic acid moieties to alginic acid allowed excellent polymeric crosslinking upon exposure to iron(III) solutions. The crosslinked polymeric matrices were explored as sustained delivery systems (Taha and Aiedeh 2000; Aiedeh and Taha 2001).

In line with our quest towards superior aliginate/divalent cations complexes, we decided to explore the thiol-zinc coordinate bond as possible crosslinking tool. Thiol moieties are known to form particularly stable complexes with divalent zinc ions (Vogler et al. 2002; Brooker and Davidson 2000). Such complexes are quite common in zinc-containing enzymes (Parkin 2000). Consequently, we envisaged the possibility of substituting a fraction of algin's carboxylic acid moieties with thiol groups followed by crosslinking the resulting polymer with zinc ions.

In the present investigation thiolated alginate-cystein (AC) matrices of variable substitution degrees were successfully prepared by attaching cysteine moieties to the carboxylic acid groups of alginic acid, as shown in Scheme 1. The conjugation process was performed employing dicyclohexylcarbodiimide (DCCI). Moreover, zinc ions were used to crosslink the resulting AC polymers leading to polymeric matrices of variable drug releasing profiles.

Thiolated polymers have recently received great interest as effective disulfide-bond forming mucoadhesives (Bernkop-Schnürch et al. 2000, 2001; Bernkop-Schnürch and Steininger 2000). On the other hand, no previous reports mentioned the use of zinc ion as ionotropic crosslinking agent in the formation of drug releasing polymeric matrices. Accordingly, our proposed use of zinc-crosslinked thiolated polymers in the area of controlled drug delivery is completely innovative.

Folic acid was used as the model drug to investigate the capacity of zinc-crosslinked AC beads in modifying folic acid release. The choice of folic acid, as the model drug, is based on fact that it is easily analyzed spectrophotometrically, and it is usually combined with zinc in dietary supplements.

2. Investigations, results and discussion

2.1. Synthesis and characterization of alginate-cysteine (AC) polymeric conjugates

The conjugation of sodium alginate to cysteine was achieved by first activating the carboxylic acid moieties with DCCI (Taha and Aiedeh 2000; Aiedeh and Taha

Scheme 1

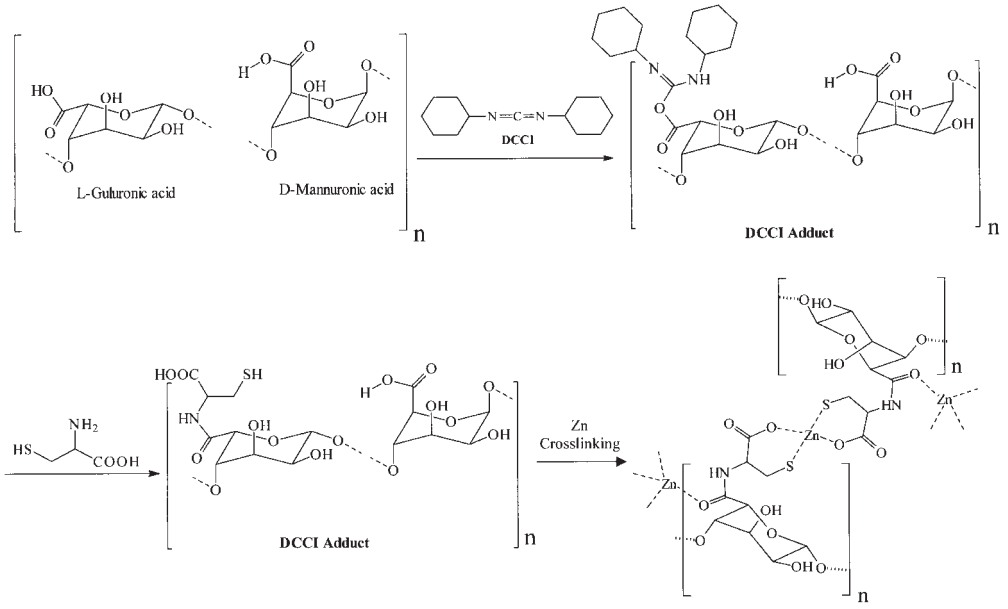


Table 1: Amounts of DCCI and cysteine hydrochloride used in preparation of different AC conjugates

Polymer	Targeted degree of substitution	DCCI g (mol)	Cysteine · HCl g (mol)
AC100	100%	10.32 (0.050)	17.56 (0.10)
AC60	60%	6.19 (0.030)	10.59 (0.06)
AC30	30%	3.10 (0.015)	5.30 (0.03)

2001), followed by quenching the reaction mixture with cysteine hydrochloride under basic conditions as shown in the Scheme. DCCI was theoretically calculated to activate 30, 60 and 100% of the available carboxylic acid groups in alginic acid to produce AC30, AC60 and AC100, respectively (Table 1). Cysteine hydrochloride was added in 2-fold excess (compared to DCCI) to force the forwardness of the reaction, particularly under the sterically hindering environment of the polysaccharide polymer (Table 1).

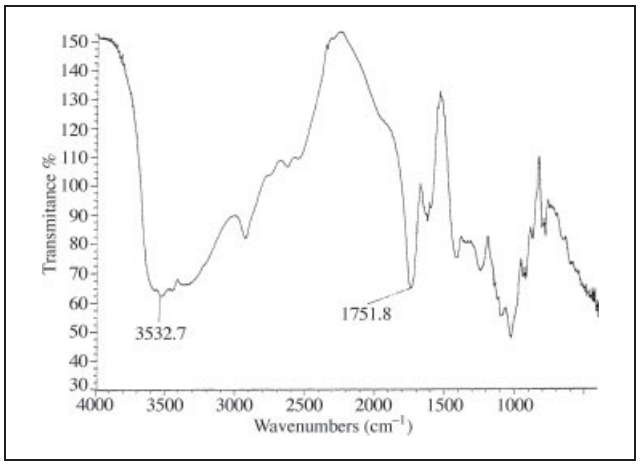


Fig. 1a: Infrared spectrum of alginic acid

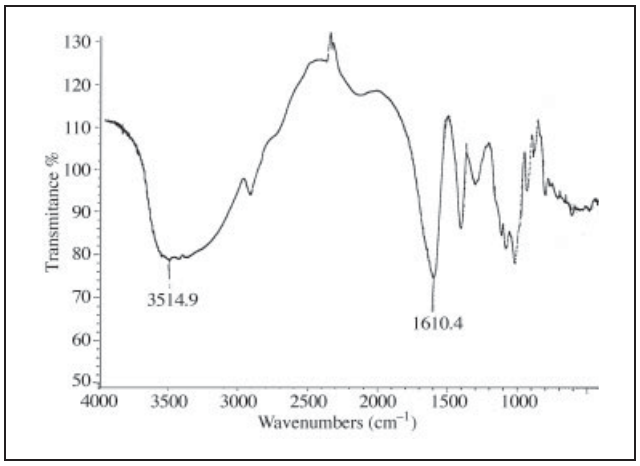


Fig. 1b: Infrared spectrum of sodium alginate

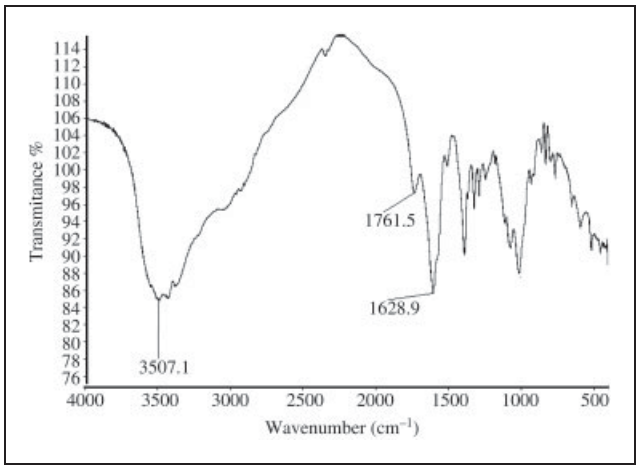


Fig. 1c: Infrared spectrum of alginate-cysteine conjugate of 30% targeted substitution degree (AC30)

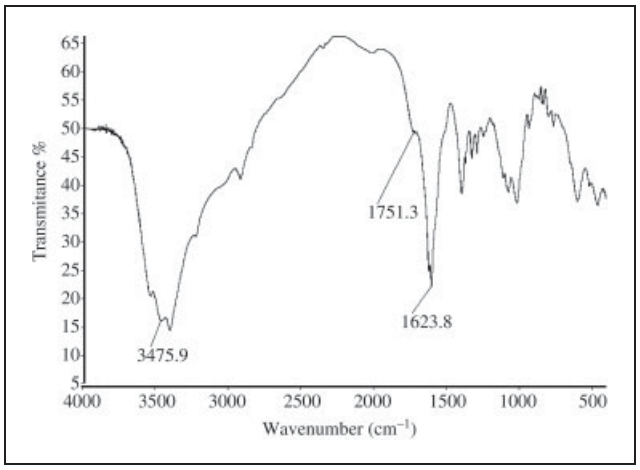


Fig. 1d: Infrared spectrum of alginate-cysteine conjugate of 100% targeted substitution degree (AC100)

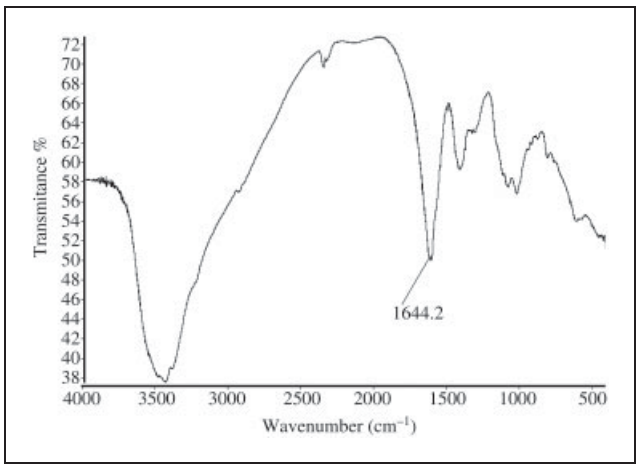


Fig. 1e: Infrared spectrum of zinc-crosslinked AC30

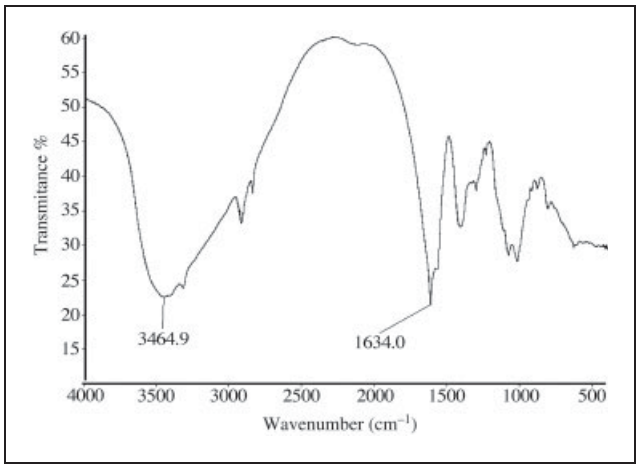


Fig. 1f: Infrared spectrum zinc-crosslinked AC100

The resulting thiolated polymers became quickly brownish upon exposure to air. It is assumed that the liability of the newly introduced thiol moieties to aerial oxidation is a reason for the brownish discoloration. Accordingly, we were prompted to add ascorbic acid as antioxidant to the reaction mixture. Furthermore, the resulting polymeric conjugates were stored in ascorbic acid solution under air-tight conditions at 4 °C. Ascorbic acid-protected polymeric conjugates were quite stable to discoloration indicating successful protection against thiol oxidation. It can be safely assumed that ascorbic acid does not interfere in the conjugation reaction as it lacks amine or carboxylic acid moieties.

The generated semi-synthetic polymers were characterized using IR spectrophotometry and iodometric titration of the thiol groups. Compared to the IR spectrum of alginic acid (Fig. 1a), it is clearly evident from the IR spectra of AC30 and AC100 (Fig. 1c and 1d) that the most drastic change that occurred upon cysteine substitution was the appearance of intense carbonyl stretching band at ca. 1625 cm^{-1}, which undoubtedly corresponds to the newly introduced amide linkages (Williams and Fleming 1997). This band seems to cover the carboxylic band at ca 1755 cm^{-1}. The apparent reduction in the intensity of the carboxylic band in AC polymers, despite the introduction of new carboxylic acid groups with cystein conjugates, is probably related to partial ionization to carboxylate in response to the basic conditions of the conjugation reaction. Carboxylate anions produce intense stretching vibrations at around 1610 cm^{-1} (Williams and Fleming 1997) as evident in the IR chart of sodium alginate (Fig. 1b).

Unsurprisingly, IR spectroscopy failed in detecting the thiol moieties in AC polymers. S−H bonds produce insignificant stretching bands at around 2700 cm^{-1} (Williams and Fleming 1997), which seem to be overshadowed by the intense broad polysacchradic hydroxyl stretching band at ca. 3500 cm^{-1} (Williams and Fleming 1997). Accordingly, we were prompted to employ quantitative iodometry to characterize thiol groups within the AC polymeric conjugates. Iodine (I_2) oxidizes thiol groups quantitatively into disulfides (Connors 1982).

Quantitative iodometry indicated incomplete conjugation of the carboxylic acid groups of alginic acid with cysteine. Table 2 shows the degree of cysteine substitution in each AC polymer. Undoubtedly, the incomplete conjugation of the alginic acid is related to the sterically hindering environment of the polysaccharide. Nevertheless, the actual degree of cystein substitution correlates well with the targeted nominal degree of substitution.

2.2. Preparation and characterization of Zn^{++}-crosslinked folic acid-loaded polymeric beads

Drug-loaded, zinc-crosslinked alginate-cystein polymeric beads (AC-Zn60 and AC-Zn30) were prepared by dropping a suspension of folic acid, dispersed in an alkaline solution of the particular AC polymer, into zinc chloride solution. The resulting beads were yellow in color, spherical biconcave in shape. They underwent approximately 10-fold reduction in their size upon drying to an average dry bead diameter of ca. 1.0 mm.

The complexation process was probed employing IR spectroscopy. However, we avoided using whole polymeric beads as IR probes, since zinc complexation is expected to take place within the vicinity of the beads' surface, which comprises a small percentage of the generated beads. Accordingly, it is anticipated that the use of whole beads will yield vibrational bands related to zinc-complexation cluttered with stronger bands resulting from non-complexed core polymer. Accordingly, zinc complexation was studied employing polymeric matrices prepared by adding the crosslinking zinc ions to vigorously stirred polymer solutions to maximize the degree of crosslinking and minimize any absorption bands related to uncomplexed polymers.

Figs. 1e and 1f show the IR spectra of zinc-crosslinked AC30 and AC100 (AC-Zn30 and AC-Zn100), respectively. It is clearly evident from both charts that the AC bands at ca. 1755 cm^{-1} and 1625 cm^{-1} corresponding to the carboxylic and amidic carbonyls, respectively (Figs. 1a, 1c and 1d), were replaced by an intense band at ca. 1640 cm^{-1} in AC-Zn polymers. This is indicative of zinc complexation to the carboxylic acid and amide moieties across the polymeric backbone, as shown in Scheme 1 (Williams and Fleming 1997).

Unfortunately, the crosslinked matrices were practically insoluble in any solvent, which made it impossible to measure the molecular weight and the degree of crosslinking by gel permeation chromatography or viscosity studies.

2.3. Folic acid dissolution profiles from various AC-Zn beads

Fig. 2 illustrates the dissolution profiles of folic acid from zinc-crosslinked AC-Zn30 and AC-Zn60 beads. Unfortunately, AC-Zn100 beads showed erratic folic acid loading and release profiles prompting us to discontinue further dissolution studies on this particular matrix.

It is clearly evident from Fig. 2 that AC-Zn60 and AC-Zn30 polymeric beads resisted drug release under acidic conditions (pH 1). However, they released most of their drug contents, i.e., around 180 mg per gram of polymeric beads, immediately upon exposure to the simulated intestinal pH (phosphate buffer 7.0).

Table 2: The degrees of cysteine substitution in different thiolated polymers as determined by iodometry

Polymer	Degree of substitution (%)	
	Targeted	Actual*
AC100	100	55.4 (±2.0)
AC60	60	29.4 (±0.9)
AC30	30	21.5 (±2.3)

* The reported values represent the results of three iodometric titration trials, the standard deviation of the three measurements is shown in brackets.

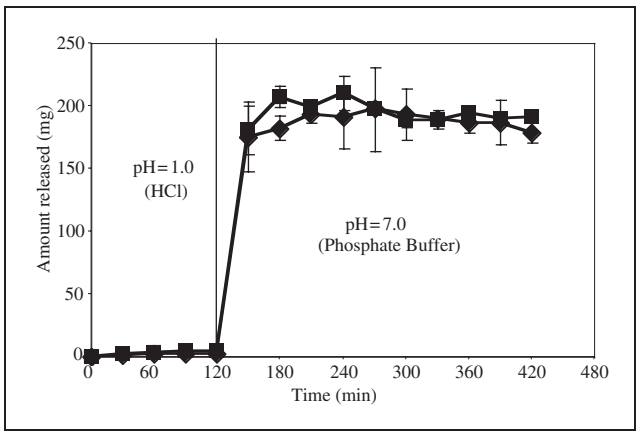


Fig. 2: Release profiles of folic acid from 1.0 g of loaded AC-Zn-60 (◆) and AC-Zn30 (■) polymeric beads. The profile represents the average of three release studies. Error bars represent the standard deviation at each point

Probably, AC-Zn matrices resisted folic acid dissolution under acidic conditions due to three factors: (i) The excessive molecular size of AC-Zn crosslinked polymers at beads' exterior renders them less water soluble and less permeable, (ii) acid-induced protonation of free polymeric carboxylic acid moieties and (iii) the enhanced hydrophobic effects of the large sulfur atoms within thiolated alginic acid. Undoubtedly, the acidic conditions of a simulated gastric environment protonate free hydrophilic carboxylate and thiolate anions into less hydrophilic carboxylic acid and thiol moieties, which resist hydration and subsequent dissolution. Furthermore, it seems that the zinc-thiolated alginic acid complexes are sufficiently stable to withstand the acidic conditions of simulated gastric environment and to maintain high crosslinked polymeric molecular size necessary for impeding drug release.

On the other hand, AC-Zn matrices released their folic acid contents immediately upon exposure to pH 7.0. The dissolution process was accompanied by disintegration of the polymeric beads into fine particles. We believe the major reason for this switch in the behavior of AC-Zn matrices, i.e., between acidic and basic environments, is related to sequestering of zinc cations by the phosphate ions within the buffer solution. Zinc ions have been reported to form insoluble chelates with phosphates (Han et al. 1994). Removal of zinc ions from the polymeric matrix seems to promote polymeric disintegration and subsequent drug release. Furthermore, pH 7.0 is expected to ionize the carboxylic acid moieties (pKa $\approx$ 4.5) and to a less degree thiol moieties (pKa $\approx$ 10).

The presence of natural zinc-sequestering agents in the intestinal fluids such as bile salts (Fini et al. 1996; Loennerdal et al. 1980) and bilirubin (Mendez-Sanchez et al. 2001) should promote similar polymeric disintegration and drug release from the AC-Zn matrices, particularly under the relatively basic conditions of the intestines.

The selective release of the model drug at pH 7.0 in the presence of zinc-sequestering agents, provides a novel strategy for enteric drug delivery, where drug release is triggered not only by a change in the pH of the digestive fluids, but also by change in their composition. In fact the drug released from AC-Zn60 and AC-Zn30 after 2 h of dissolution in acidic medium (1.5% and 2.5%, respectively) was less than the USP limits for enteric dosage forms (10%).

2.4. Zinc release from different AC-Zn matrices

Fig. 3 shows the release profile of zinc from AC-Zn30 and AC-Zn60 under acidic conditions (pH 1.0). It evident from the figure that the amount of zinc leached from the polymeric matrices did not exceed 30 mg per 1.0 g polymer. Probably, most of the released zinc comes from uncomplexed zinc at the beads' surfaces. This quantity is unlikely to cause any zinc intoxication. Furthermore, complexed zinc, i.e., in the intestines, is generally not available for absorption (Hayashi et al. 2001).

Studies in experimental animals show that whole body zinc content remains constant over a ten-fold range in dietary intakes. The same seems to be true in humans as a result of adjustments in gastrointestinal zinc absorption and endogenous fecal zinc excretion (King et al. 2000). The normal daily intake of zinc ranges from 15 to 22 mg/day for adults (WHO 1996). The acute toxicity of zinc ranges from 237 to 623 mg/kg in rats and from 86 to 605 mg/kg in mice after oral administration (Domingo et al. 1988).

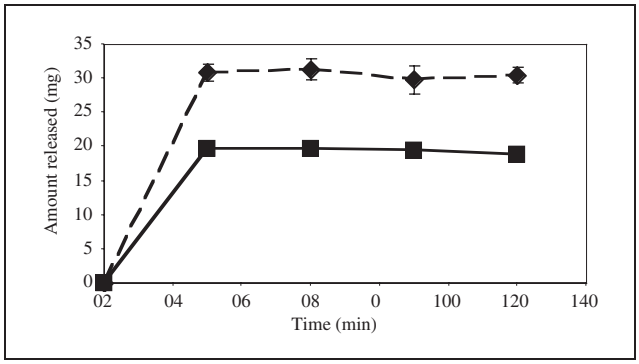


Fig. 3: The amounts of zinc released from 1.0 g of loaded AC-Zn-60 (◆) and AC-Zn30 (■) drug loaded polymeric beads under acidic conditions (pH 1.0). The profile represents the average of three release studies. Error bars represent the standard deviation at each point

3. Experimental

3.1. Chemicals

Reagent grade chemicals were purchased from the corresponding companies: sodium alginate (Hipure, Genzyme-England), dicyclohexylcarbodiimide (DCCI, Fluka-Switzerland), ascorbic acid (Sigma-Aldrich Chemie GMBH, Germany), folic acid (Sigma-Aldrich Chemie GMBH, Germany), nitric acid (Frtaron Ltd., UK), zinc chloride (C-13-H Lab. Chemicals, Nottingham-UK) and sodium hydroxide (S.D. Fine-Chem. Ltd. Boisar-India). All chemicals were used as obtained from the manufacturers without further purification.

3.2. Synthesis of alginate-cysteine (AC) polymeric conjugates (AC100, AC60 and AC30)

To a magnetically stirred solution of sodium alginate (10.40 g, 0.05 mol carboxylate) in distilled water (500 ml), ascorbic acid (10.0 g) was added. Subsequently, the pH of the overall solution was adjusted to 3–4 using nitric acid (1.5 M, 100 ml). Thereafter, dicyclohexylcarbodiimide (DCCI, quantities in Table 1) was added to the reaction mixture to achieve variable degrees of substitution. Two hours later cysteine hydrochloride (amounts in Table 1) was added to the reaction mixture. Subsequently, the pH of the reaction mixture was raised to 6 using sodium hydroxide solution (2.0 M). Two hours later the pH was further raised to 9 using the same sodium hydroxide solution. After 24 h the reaction was terminated by precipitation with nitric acid (100 ml, 1.5 M) and acetone (1000 ml). The generated precipitate was filtered and thoroughly washed with acetone (3×250 ml), ethanol (3×250 ml) and diethylether (3×250 ml). The resulting fibrous polymer was stored in an acetonic solution of ascorbic acid (2% w/v) at -4 °C. All reaction steps were conducted under nitrogen gas to avoid thiol oxidation.

3.3. Preparation of drug loaded zinc-crosslinked polymeric beads (AC-Zn60 and AC-Zn30)

A 1.0 g quantity of the particular polymer (AC30 and AC60) was washed thoroughly with water (100 ml $\times$ 3 times) and acetone (100 ml $\times$ 3 times) to remove residual ascorbic acid used during storage. Subsequently, the polymer quantity was dissolved in sodium hydroxide solution (0.1 N, 20 ml) to yield a viscous polymeric solution. Folic acid (0.5 g) was subsequently added to the generated viscous solution and the mixture was stirred over 15 min. Folic acid yielded a fine suspension upon mixing in the polymeric solution. The resulting viscous suspension was carefully dropped, using a glass dropper, into a stagnant aqueous solution of zinc chloride (1.0% w/v, 300 ml). The viscous droplets were left to cure in zinc chloride solution over 45 min to generate yellow beads. Bead preparation and curing was carried out under ambient room temperature. The beads were then collected and washed with water (3×25 ml) and left to dry at room temperature over 24 h. Upon qualitative visual inspection, the prepared beads suffered from approximately 10-fold reduction in their size upon drying and were bright yellow in color, rough-surfaced, spherical in shape, with an average dry bead-diameter of around 1.0 mm.

3.4. Characterization of different polymers

3.4.1. Infrared characterization of AC polymers and Zn-crosslinked AC polymers

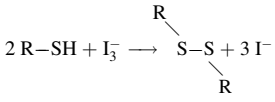
The AC matrices (AC100, AC60 and AC30) and zinc-crosslinked AC matrices (AC-Zn100, AC-Zn60 and AC-Zn30) were characterized using IR spectrophotometry recorded on a Hitachi 270-50 IR spectrophotometer using KBr disks. Zinc-crosslinked AC matrices were prepared by adding

zinc chloride aqueous solution (1.0% w/v, 20 ml) to a vigorously stirred solution of the particular AC polymer (1.0 g) in sodium hydroxide solution (0.1 N, 10 ml). The resulting white solid mass was filtered, washed thoroughly with water (3×50 ml), then washed with acetone and left to dry at room temperature over night. The dried composite matrices were crushed and KBr discs were prepared from the resulting fine powders.

3.4.2. Iodometric determination of cysteine substitution degree within AC polymers

The degree of cysteine substitution on alginic acid was determined by iodometric titration of the corresponding thiol groups of each AC polymer. The particular polymer (0.40 g, 0.26 g and 0.22 g for AC100, AC30 and AC60, respectively) was dissolved in distilled water (20 ml). Subsequently, hydrochloric acid (10 ml, 1.0 M) and starch solution (1 ml) were added to the polymeric solution. The resulting mixture was then titrated with iodine solution (0.005 M). The endpoint was visually detected by the appearance of a stable blue color (Connors 1982). The titration was repeated three times for each AC polymer. Iodine titration volumes were reported. Subsequently, the percentage of thiol moieties per total polymeric carboxylic acid groups (mole ratio) was calculated for each AC sample. Table 2 shows the percentages of cysteine substitution for each AC polymer. The titration reaction is illustrated in the following Scheme 2.

Scheme 2



The number of thiol moles (ThM) was determined from the following equation:

$$\text{ThM} = 2 \times \text{end point volume} \times \text{concentration of titration solution (0.005M)}$$

The degree of substitution (DS) was determined from the following equation:

$$\text{DS} = \frac{\text{mols of thiol}}{\text{mols of carboxylic acid (from alginic acid)}} \times 100\% \qquad (1)$$

3.5. Dissolution profiles

2.5.1. Folic acid dissolution from different polymeric beads (AC-Zn series)

A rotating basket apparatus (Erweka DTD) fitted with a 0.125 mm-stainless steel basket was used. Dry beads (0.30 g) were placed in the basket. Two buffered dissolution media were used over two subsequent stages: pH 1.0 (0.1 N HCl) for 2 h, then pH 7.0 (phosphate buffer) for 5–6 h. The dissolution mediums (900 ml) were maintained at 37 °C. The basket was rotated at 100 round/min. Samples (2 ml) were withdrawn appropriately every 30 min for the analysis of released folic acid. The withdrawn volume was immediately replaced with an equivalent volume of the fresh medium maintained at the same temperature. The absorbencies of collected folic acid samples were evaluated at the corresponding λ_{max} wavelengths, i.e., 296 and 283 nm for samples collected from simulated gastric and intestinal fluids, respectively, using a Cary 1E UV-visible spectrophotometer. Unloaded beads were used as blanks. The release experiments were repeated three times and the average released folic acid was reported. The concentrations were calculated from a appropriately drawn calibration plots (for each pH medium). The amounts released were plotted against time for each class of polymeric beads.

3.5.2. Zinc dissolution from different polymeric beads (AC-Zn series)

The dissolution apparatus and experimental conditions are as in section 2.5. Folic acid-loaded, zinc-crosslinked beads were used to characterize zinc release profiles. Zinc profiles were investigated only under acidic conditions (pH 1.0). The following procedure was performed to determine the amount of zinc released. Samples (2.0 ml) were collected every 30 min for analysis. The withdrawn volume was immediately replaced with an equivalent volume of fresh medium maintained at the same temperature (37 °C). Subsequently, the samples were analyzed for their zinc concentration (ppm) using atomic absorption spectrophotometry on a PerkinElmer 2280 spectrometer (U.S.A). The spectrophotometer was calibrated using five standard zinc solutions. Subsequently, sample zinc concentrations were converted into released amounts by multiplying by 900 (the volume of the release medium in ml). The release experiments were repeated three times and the average released zinc was reported in mg. The released amounts were plotted against time for each type of polymeric beads.

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