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Research paper

Inulin–iron complexes: A potential treatment of iron deficiency anaemia

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Abstract

The aim of this work was that to synthesize macromolecular derivatives based on inulin able to complex iron and useful in the treatment of iron deficiency anaemia. Carboxylated or thiolated/carboxylated inulin derivatives were obtained by single or double step reactions, respectively. The first one was obtained by reaction of inulin (INU) with succinic anhydride (SA) alone obtaining INU–SA derivative; the second one was obtained by the reaction of INU with succinic anhydride and subsequent reaction of INU–SA with cysteine; both derivatives were treated with ferric chloride in order to obtain the INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes. Both complexes showed an excellent biodegradability in the presence of inulinase and pronounced mucoadhesion properties; in particular, thiolated derivative INU–SA–Cys showed greater mucoadhesive properties than polyacrylic acid chosen, as a positive reference polymer, and a good iron release profile in condition mimicking the intestinal tract. These results suggest the potential employment of such systems in the oral treatment of iron deficiency anaemia or as supplement of iron in foods.

Keywords: Inulin; Iron complexes; Thiolated polymers; Mucoadhesion; Colon release

1. Introduction

Iron deficiency anaemia (IDA) is an extremely disseminate trouble involving about 1/3 of the global population. World Health Organization estimates that 46% of the world's 5- to 14-year-old children are anaemic, the great majority resident in developing country [1]. In the Third World, 56% of pregnant women are anaemic. Even in developed country such as USA, about 7.8 million of women and about 700,000 of growing children have problems of iron deficiency [2,3]. Iron is a component of proteins required for crucial cellular processes being iron-containing proteins involved in oxygen transport, ATP production, DNA synthesis, and other physiological processes [4–6]. The major processes responsible for modulating mammalian iron homeostasis are: intestinal absorption, interorgan transport and uptake, and cellular utilization [2]. Ferric iron is absorbed in the intestinal tract via a β_3 integrin and mobilferrin pathway (IMP) which is unshared with other nutritional metals. Ferrous iron uptake in the intestinal tract is facilitated by a DMT-1 (divalent metal transporter-1) pathway that is shared with manganese [7,8]. In iron deficiency, large quantities of both mobilferrin and DMT-1 are found in goblet cells and intraluminal mucin suggesting that they are secreted with mucin into the intestinal lumen where they bind either ferric or ferrous iron to maintain the iron available for absorption by the enterocytes [7,9–13]. Furthermore, it was demonstrated that colon (both proximal and distal portion) is a good site for iron absorption, even more significantly, in condition of iron deficiency since in such circumstance, iron transport systems, including DMT1, were expressed in the large intestine at high levels [7,14–18]. For these reasons, oral administration of iron can be successfully exploited to treat iron deficiency. The main drawbacks of

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oral iron administration are nausea, epigastric discomfort, diarrhoea, and constipation occurring within 1 h or two of ingestion [19,3]. These symptoms vary proportionally to the concentration of ionizable iron in the upper gastrointestinal tract and they can be reduced by taking iron with food or using a chelated form. Other problems associated with the use of iron are the low solubility of uncomplexed form and its propensity to catalyze formation of toxic oxidants. In the last years, it was demonstrated that some naturally occurring nondigestible fructooligosaccharides such as inulin, with prebiotic effect, and their products of fermentation into the colon, could have an enhancing effect in iron absorption [20–24]. The mechanisms involved in this enhancer effect can be various; among all, the production of short-chain fatty acids, due to fermentation of inulin into the colon, determines a lowering in luminal pH of the colon with consequent increase in iron solubility and absorption [20]. Considering the potential to improve iron absorption by the co-administration of fructooligosaccharides such as inulin and the need to decrease side effects caused by the conventional iron administration eventually also with chelate forms, our idea was that to design and synthesize macromolecular derivatives of inulin able to complex iron and useful for oral iron administration. To this aim two different copolymers based on inulin have been prepared: a carboxylated derivative of inulin obtained by derivatization of inulin with succinic anhydride (SA) called INU–SA. The introduction of carboxylic moieties into inulin structure should enhance the iron complexation properties of INU. Furthermore, the reduction of intraluminal pH, determined by carboxyl acid groups, should promote iron solubility [25,26,20]. Subsequently a thiolated/carboxylated derivative has been prepared by the reaction of INU–SA with cysteine (Cys) called INU–SA–Cys. The thiolated/carboxylated inulin has been ideated in order to enhance the interaction of inulin with intestinal mucosa [27] (by its interaction with mucin) with the aim to increase the residence time into the intestinal tract and to promote the absorption of iron consequently to colonic degradation of inulin backbone. The obtained INU–SA and INU–SA–Cys derivatives have been characterized and tested as iron complexing agents obtaining two different complexes INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III}.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade. Succinic anhydride (SA), inulin from Dahlia Tubers Mw ≈ 5000 Da, inulinase from *Aspergillus niger* (INU-ase), cysteine hydrochloride anhydrous >99.5% (Cys), butanol 99.5%, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride >99% (EDC), DL-dithiothreitol 99.5% (DTT) and triethylamine (TEA) were from Fluka (Italy). Anhydrous *N*,*N*-dimethylformamide 99.9% (DMF), *N*,*N*-dimethylacetamide 99.8% (DMA), orcinol 97%, D-(−)-fructose (Fru), 1,10-phenanthroline >99%, anthrone 97%, picrylsulfonic acid (TNBS), Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), D_2O (isotopic purity 99.9%), sulfuric acid 95–98%, Dowex 50W × 8 200 mesh cationic exchange resin, polyacrylic acid (Mw 5000), mucin from porcine stomach type II and Tris(hydroxymethyl)aminomethane 99.8% were purchased from Aldrich Chemical Co. (Italy). Diethyl ether, acetone, methanol, acetonitrile, acetic acid, and 2-propanol were purchased from Merck (Germany), Pullulan GPC standards were from Polymer Laboratories (Germany). Iron(III) chloride anhydrous min 98% was from Riedel-de-Haen. Silica gel 60 SIL $GIUV_{254}$ TLC plates were purchased from Macherey–Nagel.

2.2. Apparatus

^1H NMR (D_2O) spectra were obtained with a Bruker AC-250 instrument.

FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Perkin-Elmer 1720 Fourier Transform Spectrophotometer with a resolution of 1 cm^{-1}; each spectrum was recorded after 100 scans.

Molecular weights of INU–SA and INU–SA–Cys were determined by a SEC system equipped with a pump system and a 410 differential refractometer (DRI) as a concentration detector all from Waters and using as columns a Ultrahydrogel 1000 (size exclusion range 10,000–500,000) and a Ultrahydrogel 250 (size exclusion range 1000–50,000) both from Waters, 0.05 M PBS at pH 7.2 as a mobile phase, at 35 °C, flow rate of 0.6 ml/min and using Pullulan (Mw range 300–150,000 Da) as a standard.

Centrifugations were performed with Beckman Coulter Allegra X-22R equipped with a fixed-angle rotor F0850 and refrigeration system.

UV studies were performed using a Shimadzu spectrophotometer UV-2401.

High-pressure liquid chromatography (HPLC) analyses were carried out by using an Agilent 1100 Liquid Chromatograph equipped with a Rheodyne 7125 injector (fitted with a 20 μl loop) and an Agilent 1100 HPLC detector online with a computerized workstation. Column: reversed-phase C_{18} (μ Bondapak; 10 μm of 250 × 4.6 mm internal diameter, obtained from Waters). Mobile phase: PBS 0.5 M, pH 5, flow rate of 1 ml/min, λ 200 nm.

Release and degradation studies were performed in a Benchtop 80 °C Incubator Orbital Shaker model 420.

2.3. Synthesis of succinylated inulin copolymer (INU–SA)

The derivatization reaction of INU with succinic anhydride (SA) has been performed by proper modification of an already reported method [28–30].

Briefly, a proper amount of inulin was dissolved in anhydrous DMF under argon and suitable amounts of triethylamine (TEA) and succinic anhydride (SA) were added according to the following ratio $X = 0.75$ and $Y = 0.35$ indicating, respectively: X = moles of SA/moles of inulin

repeating units and $Y =$ moles of TEA/moles of inulin repeating units.

The reaction mixture was allowed to react at 25 °C under continuous stirring for 24 h. After this time, the reaction mixture was precipitated in 140 ml of a mixture of ether/acetone 2:1 v/v and centrifuged for 15 min at 10,000 rpm and 4 °C. The product was recovered, washed several times with a total of 500 ml of the same mixture of solvents and then dried under vacuum. One gram of the obtained product, dissolved in 60 ml of twice-distilled water, was passed through a Dowex 50W × 8 cationic exchange resin and resulting solution freeze-dried. The final product was obtained with a yield of 99% (w/w) based on the starting inulin (Scheme 1).

2.4. Characterization of INU–SA copolymer

The obtained copolymer was characterized by FT-IR spectroscopy, ^{1}H NMR analysis, by titration of succinic carboxyl groups after passage through a cationic exchange resin and SEC analysis.

FT-IR (KBr) spectrum showed a broad band centred at 3300 cm^{-1} (ν_{as} OH) and a strong band at 1732 (ν_{as} COO).

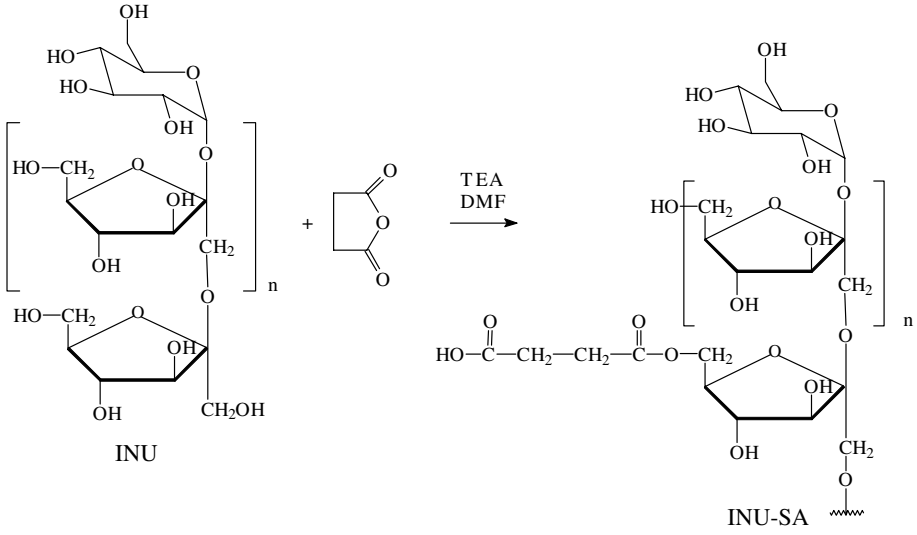
^{1}H NMR (D_2O) showed peaks at δ 2.7 (4H, s, –**CH$_2$ CH$_2$**–) 3.5–4.0 (5H, m, –**CH$_2$**–OH; **CH**–CH$_2$–OH; –CH$_2$–**CH$_2$**–O–), 4.14 (1H, t, **CH**–OH), 4.25 (1H, d, **CH**–OH). The determination of the degree of derivatization (DD_{SA} %) was performed by ^{1}H NMR and titration. By ^{1}H NMR the amount of linked succinic residues was calculated by comparing the peak integrals at δ 2.7 (–**CH$_2$CH$_2$**–) relative to succinic group, with the peaks between δ 3.5 and 4.25 relative to inulin fructose unit. The amount of succinic residues was confirmed by titration with NaOH 0.01 N after passing through a Dowex 50W × 8 cationic exchange resin. The DD_{SA} % by titration was calculated according to the Equation:

$$DD = \frac{V_{NaOH} \cdot N}{\frac{(W_{tot}) - [(V_{NaOH} \cdot N) \cdot Mw_{r.u.\ pol\ 100\%\ sub}]}{Mw_{r.u.\ start\ pol}} + (V_{NaOH} \cdot N)} \cdot 100 \quad (1)$$

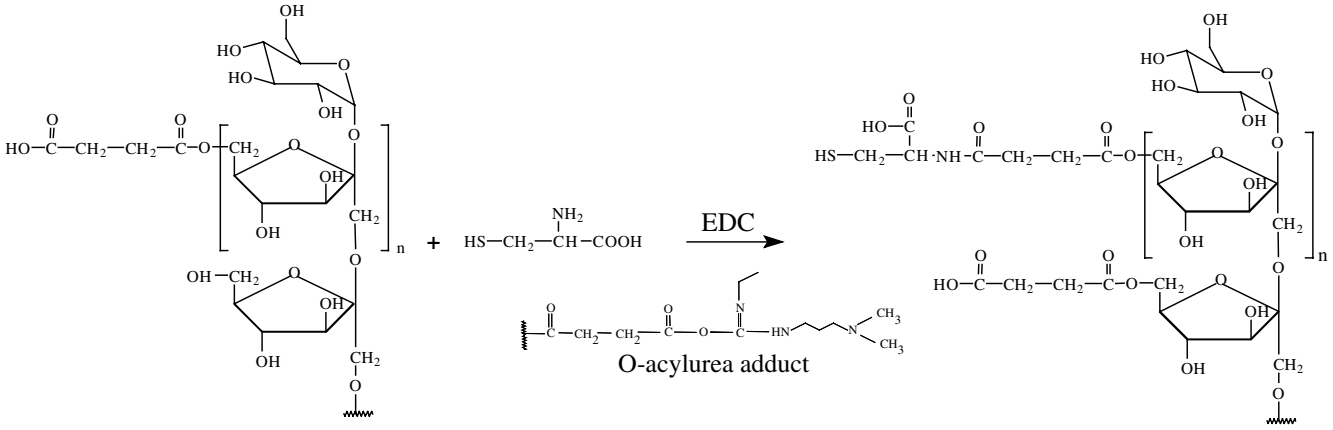
where V_{NaOH} is the volume of the base used in the titration, N is the normality of the base solution, W_{tot} is the weight of the analysed sample, $Mw_{r.u.\ pol\ 100\%\ sub}$ is the molecular weight of repeating unit of INU–SA (assuming that all the hydroxyl groups of inulin were derivatized with SA) and $Mw_{r.u.\ start\ pol}$ is the molecular weight of the repeating unit of starting inulin. DD_{SA} % obtained by both methods were in good agreement and equal to about 70 ± 2 mol % in succinic groups.

2.5. Synthesis of thiolated/succinylated inulin copolymer (INU–SA–Cys)

Five hundred milligrams of INU–SA was dissolved in 25 ml of nitrogen bubbled twice-distilled water at 25 °C and then a suitable quantity of EDC was added according to $X^1 = 1.2$ being $X^1 =$ moles of EDC/moles of inulin repeating units; the pH value of reaction mixture was regulated and maintained at 4.75, the activation reaction was carried out at 25 °C under argon for 1 h. After this time, cysteine hydrochloride was added according to $Y^1 = 2$ being $Y^1 =$ moles of Cys/moles of EDC. The reaction mixture was kept under argon for 3 h at room temperature maintaining the pH value at 5.3. After this time, the solution pH was adjusted at 8.0 and DTT (a 3-fold molar quantity in comparison with cysteine used amount) was added and the mixture stirred for 3 h. After this time the mixture of reaction was dialysed by using a dialysis membrane ROTH® Zellu Trans V series (cut off 1000 Da) against nitrogen bubbled distilled water at pH 4 for HCl and containing also 1% (w/v) of NaCl for 24 h and for further 24 h without NaCl. The collected solution was lyophilized; the product was recovered with a yield of 85% (w/w) based on the starting INU–SA (Scheme 2).



Scheme 1. Scheme of reaction between inulin (INU) and succinic anhydride (SA) to give INU–SA copolymer.



Scheme 2. Scheme of reaction between INU–SA and cysteine (Cys) to give INU–SA–Cys derivative.

2.6. Characterization of INU–SA–Cys copolymer

The obtained copolymer was characterized by FT-IR spectroscopy, ^{1}H NMR analysis, HPLC, DTNB, and TNBS assays and SEC analysis.

FT-IR spectrum did not show significant differences in comparison with INU–SA spectrum.

^{1}H NMR (D_2O) showed peaks at δ 2.7 (4H, s, –**CH$_2$CH$_2$**–), 3.5–4.0 (5H, m, –**CH$_2$**–OH; **CH**–CH$_2$–OH; –CH$_2$–**CH$_2$**–O–), 4.14 (1H, t, **CH**–OH), 4.25 (1H, d, **CH**–OH), and 2.88 (2H, s, C**H$_2$**–SH). The degree of derivatization in cysteine (DD_{Cys} %) was calculated by comparing the peak integral at δ 2.88 (2H, s, C**H$_2$**–SH) relative to cysteine group, with the integral of the peaks between δ 3.5 and 4.25 relative to inulin fructose unit. The DD_{Cys} % calculated by this method resulted to be 46 mol % of cysteine compared to inulin repeating unit or 64 mol % of cysteine compared to succinic groups bonded to inulin, being the quantity of cysteine for gram of polymer 494 μmol/g.

The purified and spectrophotometrically characterized product was subjected to HPLC and Ellman's assay. In particular, 10 mg of the sample was extemporaneously dissolved in 1 ml of pH 5.0 PBS, injected and the eluate was detected at 200 nm. No absorption of inulin, solvent, succinic anhydride or INU–SA was detected at 200 nm, while cysteine and INU–SA–Cys showed a peak of absorption at 200 nm proportional to the solution concentration. The concentration value was extrapolated by means of a calibration curve obtained by using standard solutions of cysteine in the range of concentrations 1–0.01 mg/ml ($y = 0.0006x - 0.0011$; $R^2 = 1$). The quantity of cysteine as resulted from HPLC studies was 540 μmol/g. Ellman's reagent was prepared by dissolving 8 mg of DTNB plus 41 mg of sodium acetate (2 and 50 mM, respectively) in 10 ml of twice-distilled water; samples were prepared as a 10 mg/ml solution in twice-distilled water. The solutions to be analysed were obtained by mixing 100 μl of 1 M Tris buffer solution (pH 8) plus 50 μl of DTNB/Na acetate solution plus 10 μl of the sample solution and diluting to a final volume to 1 ml with twice-distilled water; all the solutions were prepared extemporarily and analysed by UV at 412 nm.

Moreover, in order to confirm if all the detected cysteine was bonded to INU–SA, the TNBS assay was performed. 20 μl of TNBS was added to 25 μl of a 2 mg/ml solution of sample (INU–SA–Cys) solubilised in a tetrahydroborate 1 M solution and diluting to a final volume of 1 ml with twice-distilled water. The UV wavelength was fixed at 420 nm.

2.7. Studies of interaction between INU–SA or INU–SA–Cys derivatives and mucin by transmittance measurements

Ten milligrams of mucin was dispersed in 10 ml of PBS, pH 6.8 (0.1%,w/v), and then this dispersion was allowed to hydrate at 37 °C for 24 h. Then, 10 mg of polyacrylic acid, INU–SA or INU–SA–Cys was dispersed in mucin dispersion and after 2, 8, or 24 h sample transmittance was measured at 650 nm.

2.8. Preparation of INU–SA and INU–SA–Cys complexes with $FeCl_3$ (INU–SA–Fe^{III}) and INU–SA–Cys–Fe^{III}

One hundred milligrams of INU–SA or INU–SA–Cys derivatives was dispersed in 150 μl of twice-distilled water and after few minutes 1 ml of a 0.5 M $FeCl_3$ solution was added (molar ratio $FeCl_3$/U.R. inulin = 1.2). A red–yellow gel was formed in about 2 min. The reaction was allowed to proceed at room temperature for 24 h.

After this time, resulting gels were washed with a 0.001 N HCl solution, then with twice-distilled water until neutral pH. Finally, the recovered products were freeze-dried.

Dried materials characterized by FT-IR showed two strong peaks at 1600 and 1450 cm^{-1} relative to asymmetric and symmetric stretching of carboxylate group implicated in Fe^{III} coordination.

2.9. Quantification of Fe^{III} amount in INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes

Ten milligrams of dried INU–SA–Fe^{III} or INU–SA–Cys–Fe^{III} complexes was dispersed in 2 ml of HCl 1N in

order to break the complexes and make soluble all released iron. After 24 h the samples were filtered and the total iron amount was evaluated by the 1,10-phenanthroline method. For 1,10-phenanthroline assay, following solutions have been prepared: (1) the reducing solution mixing 12.5 ml of fuming HCl and 5.5 g of ascorbic acid adjusting the final volume at 500 ml with twice-distilled water, (2) the chromogen solution by mixing 68 g of sodium acetate and 45 mg of 1,10-phenanthroline in 250 ml of twice-distilled water. Then, 100 μl of each hydrochloric solution was adjusted at 1 ml with twice-distilled water and added to 1 ml of reducing solution. After 30 min, 1 ml of chromogen solution was added and the UV detection was performed at $\lambda = 411$ nm. The calibration curve was performed by using standard solutions of $FeCl_2$ in twice-distilled water ($y = 0.00028x - 0.00003$; $R^2 = 0.99913$). The iron amount resulted to be 175 mg/g of complex for INU–SA–Fe^{III} and 160 mg/g of complex for INU–SA–Cys–Fe^{III}.

2.10. In-vitro chemical degradation studies of INU–SA and INU–SA–Cys copolymers and their complexes with Fe^{III}

Aliquots (10 mg) of INU–SA or INU–SA–Cys copolymers or their complexes with Fe^{III} were added to 4 ml of degradation media, i.e. HCl 0.1 N (pH 1.0) or phosphate buffer solution pH 6.8 and incubated at 37 ± 0.1 °C under continuous stirring (100 rpm). At proper time intervals (1 or 2 h for experiments at pH 1.0 or 24 h for experiments at pH 6.8) samples were filtered and the solutions containing the degraded copolymer portion were analysed by using two different methods: (1) TLC analyses with orcinol as a detector were used for soluble derivatives (INU–SA and INU–SA–Cys); (2) anthrone method was used for insoluble complexes (INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III}).

TLC method: degradation media of INU–SA or INU–SA–Cys were chromatographed in a silicagel 60 TLC plate for 1h using a mixture of butanol/acetic acid/water, 3:3:2 v/v/v as a mobile phase and a 0.5 mg/ml fructose solution as a reference. After the developing time of the samples plate was dried with hot air, sprayed with orcinol and again dried over a hot plate.

Anthrone method: 50 mg of anthrone was solubilised in 100 ml of a mixture sulfuric acid/water 2.5:1 v/v and then, 200 μl of this solution was added to a solution containing 700 μl of water and 100 μl of INU–SA–Fe^{III} or INU–SA–Cys–Fe^{III} degradation medium; the mixture was kept at 100 °C in hot water for 10 min. After this time the solution was allowed to cool at room temperature and analysed at $\lambda = 625$ nm.

Each experiment was performed in triplicate and the results were in agreement within ±2% standard error.

2.11. In-vitro enzymatic degradation studies of INU–SA,INU–SA–Cys copolymers and their complexes with Fe^{III}

Aliquots (10 mg) of INU–SA or INU–SA–Cys copolymers or their dried complexes with Fe^{III} were incubated with 5 ml of citrate buffer solution at pH 4.7 in the absence or in the presence of inulinase (final enzyme concentration 10 U/ml), under continuous stirring (100 rpm) at 37 ± 0.1 °C for 24 h. Enzyme solutions were prepared immediately before the experiments, enzyme activity was evaluated considering that 1 U of inulinase corresponds to the amount of enzyme which releases 1 μmol of reducing sugar (measured as fructose) per minute at pH 4.1 and 37 °C from inulin, as reported by Fluka specifications. At proper time intervals the degradation of the samples was evaluated by using the above-reported procedure. Each experiment was performed in triplicate and the results were in agreement within ±2% standard error.

2.12. Iron release from INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes

Aliquots (10 mg) of INU–SA–Fe^{III} or INU–SA–Cys–Fe^{III} dried complexes were added to 0.1% (w/v) mucin dispersion in Tris buffer at pH 6.8 containing 10 U/ml inulinase. The same experiment has been also performed in Tris buffer alone, in the absence of mucin and inulinase. After proper time intervals (1, 3, 7, or 24 h) samples were centrifuged for 20 min, 4 °C at 10,000 rpm and supernatant was separated from precipitate. To this supernatant 1 ml of NaOH 1N was added and the solution stirred for 24 h. The so-obtained $[Fe(OH)_3]_6$ precipitate was separated from supernatant by centrifugation; 1 ml of HCl 1 N was added to the precipitate while 2 ml of HCl 1 N was added to the supernatant. The obtained solutions were subjected to 1,10-phenanthroline in order to quantify the released iron.

In particular, 100 μl of each release solution was adjusted at 1 ml with twice-distilled water and added to 1 ml of reducing solution. After 30 min, 1 ml of chromogen solution was added and the UV detection was performed at $\lambda = 411$ nm. The calibration curve was performed by using standard solutions of $FeCl_2$ in twice-distilled water ($y = 0.00028x - 0.00003$; $R^2 = 0.99913$).

3. Results and discussion

In this paper a new approach to delivery iron ions into the gastrointestinal tract was designed. In order to obtain novel materials able to form complexes with iron ions useful as potential systems for the oral treatment of iron deficiency anaemia, we have performed suitable chemical modifications on inulin backbone. Inulin, a fructose polymer, was chosen for its colon biodegradability, biocompatibility, prebiotic properties and for the role played by itself and its degradation products in iron absorption [20–24].

Taking into account that carboxyl groups are involved in the chelation of a wide variety of cations [25,26,19] and their presence could facilitate iron solubilisation by pH lowering, inulin was derivatized with succinic anhydride (SA) in organic phase (anhydrous DMF) for 24 h at 25 °C by using TEA as a catalyst, in order to introduce

in its backbone pendant carboxyl groups (Scheme 1). The INU–SA copolymer, obtained with a quantitative yield based on the starting inulin, has been purified by using a cationic exchange resin. This is an important step because in this way it is possible to obtain a sample with all carboxylic groups in the non-dissociated form. As a consequence, the derivatization degree in succinic residues (DD_{SA}) of INU–SA has been determined by an easy titration procedure of carboxylic groups of SA residues (see experimental part).

^{1}H NMR analysis of INU–SA derivative (Fig. 1) showed a new peak, in comparison with native inulin, at δ 2.7 (4H, s, –CH_2–CH_2–) that confirms the introduction of succinic groups in this derivative. The DD_{SA} values, calculated both by ^{1}H NMR (see experimental part) and titration, resulted to be 70 ± 2 mol % in SA residues.

FT-IR spectrum of INU–SA (Fig. 2) showed a strong band at 1732 cm^{-1} attributable to stretching of C=O group due to the presence of the ester bonds of SA residues linked to inulin as well as to the introduction of carboxyl groups of SA.

In addition, INU–SA derivative resulted to be very water soluble at 25 °C unlike the native inulin, reasonably for the presence of carboxyl groups that increase solvatation properties of copolymer.

In a second step a thiolated derivative of INU–SA has been developed. This approach was attempted in order to enhance the bioadhesive properties of INU–SA derivative thanks to the interactions of thiol groups with cysteine moieties of mucin. This interaction could either enhance the residence time of polymeric system along the intestinal

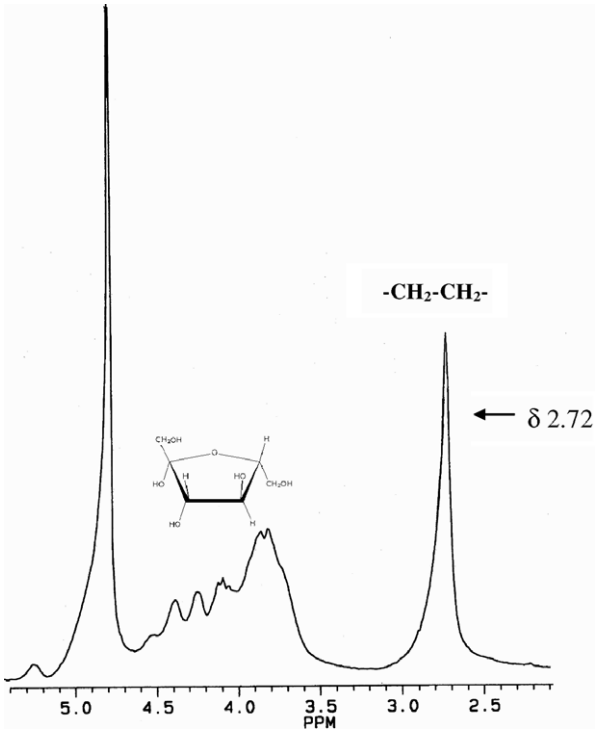


Fig. 1. ^{1}H NMR spectrum of INU–SA derivative.

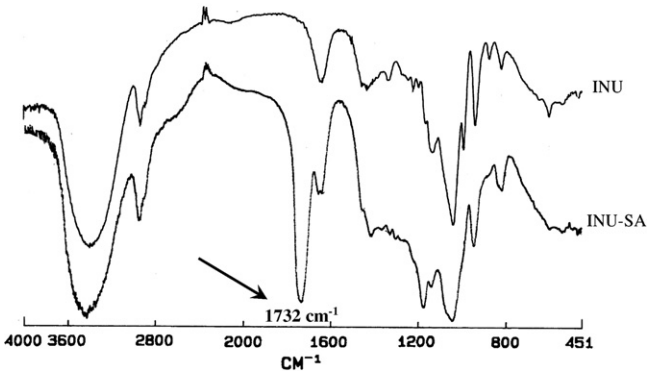


Fig. 2. FT-IR spectra of inulin (INU) and INU–SA derivative.

tract or improve iron absorption because of the fundamental role played by mucin in the iron uptake in the intestinal tract [13]. Furthermore, it was demonstrated that thiol groups might contribute to iron complexation and in this way improve the complexing performance of INU–SA [31].

In order to obtain the thiolated derivative, INU–SA was derivatized with cysteine. INU–SA derivative was allowed to react with cysteine in nitrogen bubbled twice-distilled water *via* carbodiimide activation. In particular, the EDC *O*-acylurea adduct obtained [32] between EDC and INU–SA activated carboxylic undergoes the nucleophilic substitution by –NH_2 group of cysteine (Scheme 2).

To ensure a low oxidation of thiol groups, the synthesis was carried out in degassed water and after reaction time, DTT was added to the mixture in order to reduce all the oxidized thiol groups. The yield of this reaction, calculated after dialysis, was 85% (w/w) in comparison with starting INU–SA. The characterization of the final derivative was performed by different techniques such as FT-IR and ^{1}H NMR analyses. FT-IR spectrum of INU–SA–Cys did not show significant differences in comparison with INU–SA spectrum, probably owing to peak overlay phenomena. ^{1}H NMR spectrum (Fig. 3) showed a well-defined peak at δ 2.88 relative to –C**H**$_2$–SH protons of cysteine group confirming the presence of cysteine linked to INU–SA copolymer. The DD_{Cys} % calculated by ^{1}H NMR (see experimental part) resulted to be 46 mol % of cysteine compared to inulin repeating unit or 64 mol % of cysteine compared to succinic groups bonded to inulin, being the quantity of cysteine for gram of polymer 494 μmol/g.

HPLC studies were performed in order to quantify the amount of bonded cysteine. In particular, considering that bonded cysteine shows a well-definite and reproducible peak at $\lambda = 200$ nm and that inulin, SA and INU–SA derivative analysed in the same experimental conditions showed only very low value of absorbance, it is useful to exploit HPLC to quantify linked cysteine in INU–SA–Cys copolymer that resulted to be equal to 540 μmol/g. This result was in good agreement with that obtained by ^{1}H NMR studies (494 μmol/g). A further quantification by Ellman's reagent confirmed these data.

Beside, the TNBS assay was performed in order to determine the possible presence of the free cysteine in the

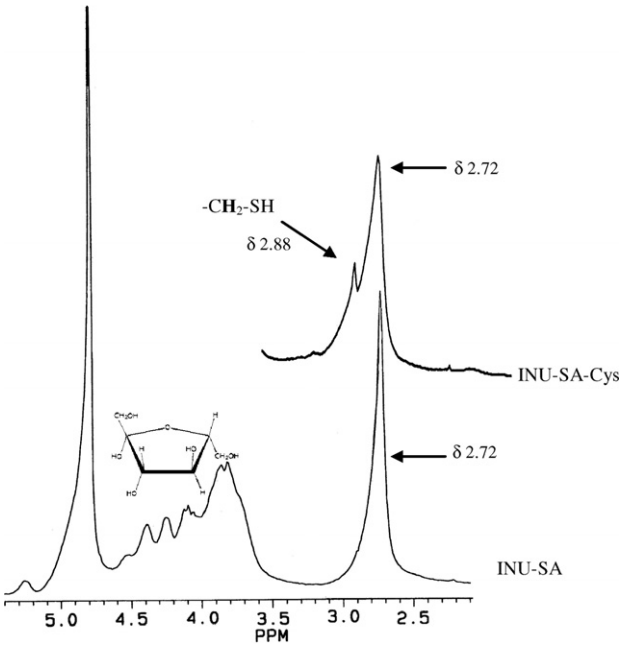


Fig. 3. ^{1}H NMR spectra of INU–SA and particular of –CH_2–SH peak of INU–SA–Cys derivative.

INU–SA–Cys copolymer by the reaction of its primary NH_2 group. The performed assay does not show absorption of the samples confirming the absence of free cysteine (as an impurity) and confirming the effective linking of all cysteine molecules to INU–SA–Cys derivative.

Furthermore, SEC analyses were performed in order to determine the average weight molecular weights of INU–SA and INU–SA–Cys derivatives (Fig. 4 and Table 1):

As shown in Table 1, INU–SA and INU–SA–Cys molecular weights are higher if compared with that of starting inulin thus confirming that the used reaction conditions do not alter the integrity of polymeric backbone; this assumption is further confirmed by the low values of polydispersity index of the copolymer.

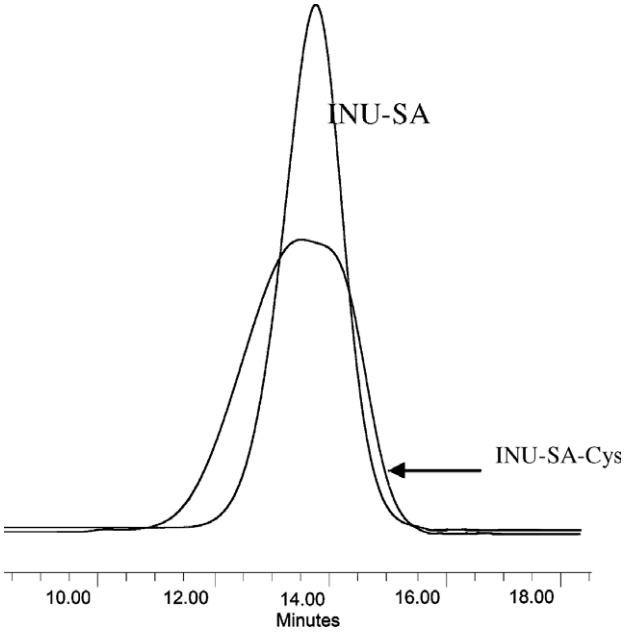


Fig. 4. SEC chromatogram of INU–SA and INU–SA–Cys derivatives.

Table 1
Molecular characterization parameters of INU based copolymers

Sample	Mw	Polydispersity index (Mw/Mn)	DD_{SA} (%)	DD_{Cys} (%)	Yield (%)
INU–SA	7600	1.20	70	0	99
INU–SA–Cys	9700	1.68	70	46	99

Mw inulin = 5000.

In order to verify the chemical stability of INU–SA and INU–SA–Cys derivatives, chemical hydrolysis studies were performed incubating them in HCl 0.1 N (simulated gastric fluid) or PBS pH 6.8 (simulated intestinal fluid). All the samples showed a great stability in both experimental conditions simulating the gastro-intestinal fluids. In particular, both incubations (pH 1.0 or 6.8) for two materials were analysed by using the TLC method; no spots, related to degradation products, were detected after treatment of the samples with orcinol, used as a fructose detector (data not showed).

Furthermore, studies of enzymatic degradation were performed with inulinase at the optimum pH value (pH 4.7) for the activity of this enzyme; in all experiments, INU–SA and INU–SA–Cys derivatives showed a complete degradation by inulinase depending on degradation time (Table 2).

No degradation was showed when samples were incubated in the above-reported condition, but in the absence of inulinase (data not showed).

The excellent enzymatic biodegradability confirms that these inulin derivatives could be used as colon biodegradable delivery systems.

In the transit through the gastrointestinal tract, thanks to the presence of appropriate functional groups, a polymeric system establishes different types of interaction with mucosal layer, including bioadhesion phenomena.

Bioadhesion is one of the most important factors that allows to achieve a prolonged interaction between a polymeric system and a biological surface [33–36]. Many examples in the literature show the relationship between the presence of specific substituents, such as carboxyl or hydroxyl groups, in the polymeric repetitive unit and bioadhesive properties; furthermore, thiolated polymers exhibit enhanced bioadhesive properties due to the interaction

Table 2
Enzymatic degradation of INU–SA and INU–SA–Cys samples: qualitative evaluation by TLC/orcinol

	1 h	2 h	8 h	24 h
INU–SA	–	+–	+	+
INU–SA–Cys	–	+–	+	+

–, no spot.
+–, medium-high intensity spot.
+ high intensity spot (like inulin).

between polymeric thiol groups and cysteine moiety of mucin [27]. Therefore, with the aim to have a preliminary information about the potential bioadhesive properties of INU–SA and INU–SA–Cys derivatives, interaction studies between these copolymers and mucin were performed by transmittance studies (a method already employed successfully by other authors) [37,38] employing polyacrylic acid, a known bioadhesive polymer, as a positive control [39]. Since it has been well proved the influence of molecular weight on bioadhesive properties, the used polyacrylic acid had a molecular weight near those of both INU–SA and INU–SA–Cys derivatives (i.e. MW ≈ 5000) [33]. In particular, INU–SA, INU–SA–Cys or polyacrylic acid was dispersed in a 0.1% (w/v) mucin dispersion and after 2, 8, or 24 h the transmittance values of the polymer–mucin mixture have been read at $\lambda = 650$ nm. Greater is the interaction between the polymer and mucin, lower is the transmittance value of the tested sample (greater UV–vis scattering) due to the formation of macro-aggregates [37,38]. As shown in Fig. 5 at all chosen time intervals, INU–SA or INU–SA–Cys/mucin systems show transmittance values lower than that of polyacrylic acid, i.e., they show an interaction with mucin stronger than that of the positive reference. Furthermore, INU–SA–Cys shows after 2 and 8 h transmittance values lower than INU–SA, whereas, after 24 h, the transmittance value of INU–SA and INU–SA–Cys systems is about the same. It is reasonable to think that INU–SA–Cys derivative interacts more quickly with mucin (according to the presence of cysteine groups) if compared with INU–SA derivative, but, for a prolonged time (24 h) both samples are able to interact efficiently with mucin.

Taking into consideration the good bioadhesive properties of INU–SA and INU–SA–Cys derivatives, their good resistance towards hydrolytic degradation and their complete degradation in the presence of inulinase together with potential of inulin in improvement of iron ion absorption, the subsequent step of this work has been that to employ carboxylated and carboxylated/thiolated inulin copolymers as iron complexing agents for a potential treatment of iron deficiency anaemia. The source of iron was ferric chloride. The addition of ferric chloride to INU–SA or INU–SA–Cys derivatives leads to the formation of red–orange gel-like product. After purification and freeze-drying, the final products were obtained as red powders. FT-IR analysis of both products revealed the appearance of new peaks related to the formation of INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes as shown in Figs. 6 and 7.

In particular, FT-IR spectra of INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes showed two strong bands at 1600 and 1450 cm^{-1} relative to asymmetric and symmetric stretching of carboxylate groups after complex formation [25]. It is important to underline that since complexes' formation takes place at a very low pH value (pH 1.02), the bands related to carboxylate groups, showed in FT-IR spectra, are due to the complex formation.

Chemical and enzymatic degradation studies were also performed for INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} systems. In these experiments, the anthrone method was used for the determination of degradation products. This method allows us to determine quantitatively the fructose resulting from degradation of investigated complexes.

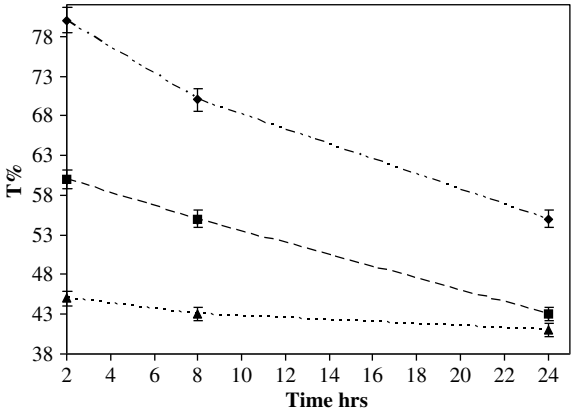


Fig. 5. Interaction studies between mucin and INU–SA (-■-), INU–SA–Cys (-▲-) or polyacrylic acid (-♦-).

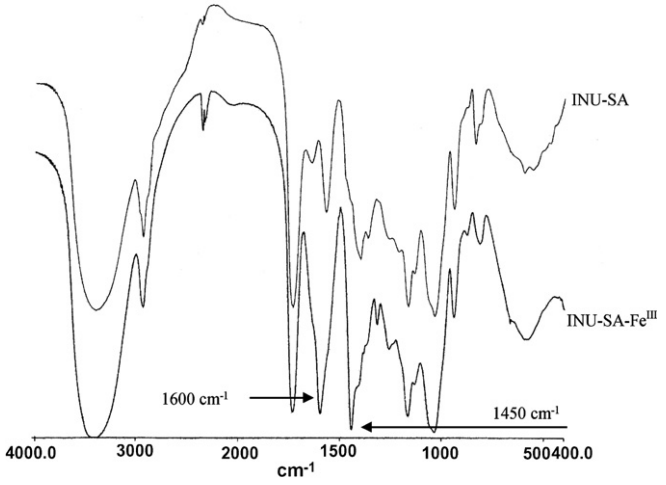


Fig. 6. FT-IR spectra of INU–SA derivative and INU–SA–Fe^{III} complex.

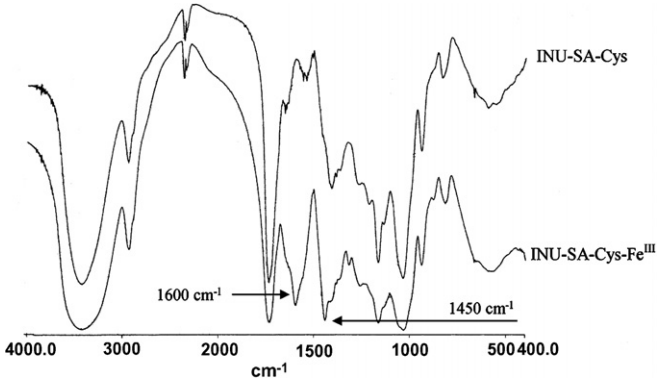


Fig. 7. FT-IR spectra of INU–SA–Cys derivative and INU–SA–Cys–Fe^{III} complex.

Table 3
Enzymatic degradation of INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} samples: quantitative determination by anthrone method

	(mg/ml) of detected fructose	(%) of degradation[a]
INU	1.98	99
INU–SA–Fe^{III}	1.46	90
INU–SACys–Fe^{III}	1.47	87.7

[a] Quantity % of detected fructose related to total sample weight.

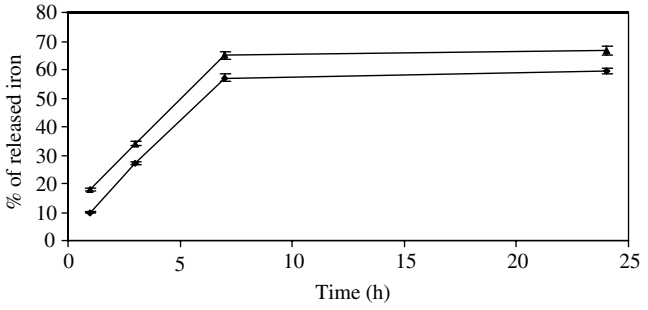


Fig. 8. Iron release from INU–SA–Fe^{III} (-♦-) and INU–SA–Cys–Fe^{III} (-▲-) complexes in Tris buffer pH 6.8 in the presence of inulinase 10 U/ml and mucin 0.1% (w/v).

INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} did not show chemical degradation after incubation until 2 h with HCl 0.1 N solution and until 24 h with phosphate buffer solution pH 6.8 (data not showed), whereas they had undergone an almost complete degradation in the presence of inulinase (Table 3) like INU–SA and INU–SA–Cys derivatives. These are very attractive results because it confirmed the resistance of INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes as well as INU–SA and INU–SA–Cys derivatives towards chemical hydrolysis, as a consequence, these systems could pass through the gastric and duodenal tracts without appreciable chemical hydrolysis, unlike starting inulin that, as known, undergoes acidic hydrolysis [40]. On the contrary, in the presence of inulinase, our systems are degraded and are potentially able to release iron ions.

Subsequently, iron release studies from INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes were performed in Tris buffer pH 6.8 in the presence of mucin and inulinase (simulated intestinal conditions) (see Fig. 8).

As can be seen in Fig. 8 both INU–SA–Fe^{III} and INU–SA–Cys–Fe^{III} complexes are able to release about 60–70% of complexed iron in simulated intestinal conditions. On the contrary, when release studies were performed in pure buffer in the absence of both inulinase and mucin no release of iron was found (data not shown).

4. Conclusions

This work proposed a new approach to delivery iron ion into intestinal tract designing new macromolecular chelating systems based on inulin. The choice of inulin as material to design a iron delivery system was based on its great biocompatibility and biodegradability into the intestinal tract. Furthermore, the formation of complexes with iron ion could assure the presence of the Fe^{III} in a more available form, as reported in the literature for complexed forms of iron. Both obtained INU–SA and INU–SA–Cys derivatives showed very good mucoadhesion properties due to the presence of specific groups such as carboxyl and thiol functions able to interact with mucin. Both copolymers showed to be able to complex Fe^{III} and to form gel systems with high chemical stability in gastrointestinal tract but excellent enzymatic degradability by inulinase and a very good iron release profile in condition mimicking the intestinal fluid.

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