Nonabsorbable Iron(III) binding polymers: synthesis and evaluation of the chelating properties.

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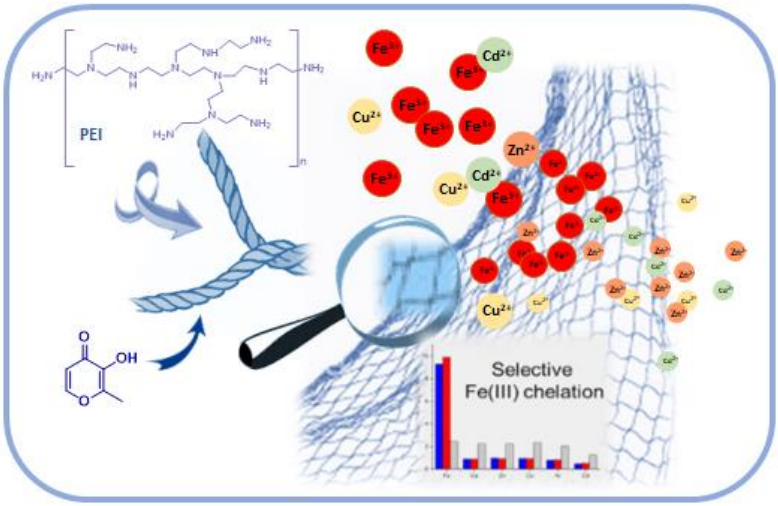
Graphical Abstract



Highlights

- Polyethylenimine (PEI) and Carboxymethyl cellulose (CMC) polymers were functionalized with 3-hydroxypyridin-4-one Fe(III) binding moiety.
- PEI derivatives were prepared using maltol and microwave irradiation.
- PEI derivatives were higher selective toward Fe(III) ion in simulated intestinal fluid.
- Both PEI and CMC derivatives did not alter the integrity of intestinal enterocytes.

Abstract

Iron is a key micronutrient essential for many biological events. While iron deficiency can lead to anemia, supplementation with oral iron often ends up with enteral iron overload, a critical gastrointestinal (GI) burden linked to the increased risk of dysbiosis, infections and often associated with colorectal cancer. Iron chelation therapy is clinically used to reduce pathological systemic iron overload by established low molecular weight iron chelators. As drawbacks, these drugs present low pharmacokinetic profiles and several toxicities, leading to relatively high rates of adverse effects. To overcome these issues, the prevention of iron accumulation in the GI tract by non-absorbable iron binding polymers could represent an alternative still underexploited approach. Here, we present the development of a series of insoluble polymeric Fe(III) chelators. These innovative compounds have been obtained by the conjugation of 3-hydroxypyridin-4-one Fe(III) chelating moiety with branched

Polyethyleneimine (PEI) and Carboxymethyl cellulose (CMC). In vitro binding studies indicated that the Fe(III) chelating capacity depends on the nature of the polymer. In particular, PEI derivatives possess higher selectivity toward Fe(III) in simulated intestinal fluid preserving the integrity of intestinal enterocytes, representing thus promising compounds in the development of iron chelators.

Keywords

Fe(III) binding polymers, N-alkyl-3-hydroxypyridin-4-one, polyethyleneimine, carboxymethyl cellulose, iron overload.

1.Introduction

Iron is a key element that is essential for many biological events, such as redox processes, oxygen transport, and DNA synthesis.[1] In mammals, iron is uptaken in the gastrointestinal tract, while its excretion is limited. As a consequence, iron levels are almost totally controlled by the absorption mechanism.[2] A high level of GI iron is typically observed during oral iron therapy to treat iron deficiency anemia (IDA). The virulence of pathogenic bacteria is indeed stimulated by Fe-rich environment, since dysbiosis and related comorbidities are driven by 'iron piracy'[3]. Pathogenic bacteria prevail and their virulence stimulated in iron-rich environment. While iron is absorbed on the duodenum, the excess luminal iron promotes dysbiosis[4] while fuels chronic pathologies such as colorectal cancer (CRC) and inflammatory bowel diseases (IBD) [5].

Nowadays, the only three iron chelators in clinical treatment are small molecules, *i. e.* deferiprone[6] **1**, desferasirox[7] **2**, and desferrioxamine[8] **3** (Figure 1). As drawbacks, these drugs usually present low pharmacokinetic profiles and several toxicities, leading to relatively high rates of adverse effects. An alternative approach is the prevention of iron accumulation within the GI tract by iron binding polymers (IBPs).[9] IBPs show reduced toxicities, and better pharmacokinetic profiles with respect to low MW iron chelators. Furthermore, oral administration of non-absorbable IBPs, which selectively sequester iron from the GI tract, could be used to promote the excretion of intestinal iron and in the prevention of the absorption of the dietary iron after chelation therapy.[10] Pre-requisites of non-absorbable IBPs are high affinity and binding activity toward iron, but also high selectivity.

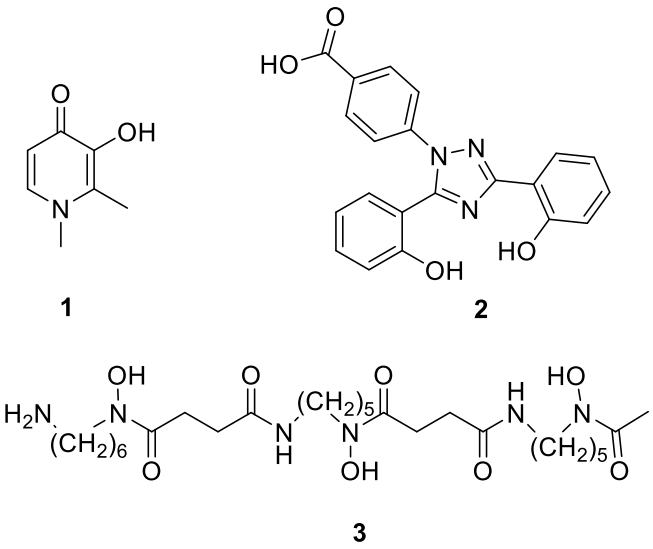


Figure 1. Iron chelators in clinical use.

Here, we propose the development of different insoluble IBPs characterized by the presence of the 3-hydroxypyridin-4-one (HPO) ligand from deferiprone linked to two different polymers: branched

Polyethylenimine (PEI) and Carboxymethyl cellulose (CMC). Atomic absorption spectroscopy experiments indicated that all the synthesized polymers present high binding activity toward Fe(III). Moreover, in simulated intestinal fluid PEI derivatives possess higher selectivity toward Fe(III) than CMC one. Finally, they do not alter the integrity of intestinal enterocytes, as assessed using human intestinal Caco-2 cell monolayer as model. These innovative compounds represent thus promising compounds in the development of non-absorbable IBPs.

2.Experimental section

2.1 Materials

Chemicals were obtained from Sigma Aldrich (Italy) and used without further purification. For the preparation of metals solutions were used metals chlorides. Melting points were determined in a Stuart Scientific melting point apparatus in open capillary tubes and are certified. Infrared spectra were recorded on a FT-IR spectrometer Perkin-Elmer 16 PC.

2.2 General procedure for the synthesis of 3-hydroxypyridin-4-one derivatives

A solution of maltol (2g, 15.8mmol) and amine (31.6 mmol, Table 1) in water (10mL) was heated in microwave instrument at 110°C for 2h (500-600W). The solution, transferred in a flask, was stirred overnight in the presence of 2g of activated charcoal. After filtration the solution was concentrated at reduced pressure and the crude was washed with *n*-hexane and diethyl ether. The resulted thick oil was crystallized by appropriate solvent (see Table 1).

Table 1. Crystallization solvent, melting point, yield and NMR characterizations of compounds **5-8**

	R_1	**Crystallization Solvent**	**M. p. °C**	**Yield %**	**^{1}H and ^{13}C NMR characterizations**
5	$(CH_2)_3NH_2$	2-propanol	168-9 (168-70 lit.)[11]	70	^{1}H NMR (300 MHz, D_2O): δ 7.41(d, J = 6.32 Hz, 1H); 6.34(d, J = 6.32 Hz, 1H); 3.98-4.03 (m, 2H); 2.68–2.73 (m, 2H); 2.28 (s, 3H), 1.84-1.89 (m, 2H). ^{13}C NMR (75 MHz, D_2O): δ 170.4; 149.2; 134.8; 112.2; 100.3; 52.3; 37.2; 30.5; 11.8.
6	$CH_2CH=CH_2$	H_2O	134-6 (136 lit)[12]	72	^{1}H NMR (300 MHz, $CDCl_3$): δ 7.23(d, J = 7.41 Hz, 1H); 6.41(d, J = 7.41 Hz,1H); 5.85-5.97 (m, 1H); 5.27-5.32 (m, 1H); 4.92-4.99 (m, 1H); 4.46-4.49 (m,2H); 2.35 (s,3H).

					^{13}C NMR (75 MHz, $CDCl_3$): δ 169.7; 146.4; 137.2; 132.1; 128.9; 118.4; 111.6; 55.6; 12.1.
7	$CH_2C_6H_5$	2-propanol	199-200 (201-3 lit)[13]	80	^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30-7.45 (m, 4H); 7.27(d, J=7.3 Hz, 1H); 6.99-7.33 (m, 2H); 6.45 (d, J=7.3 Hz, 1H); 5.20 (bs,1H); 5.09 (s, 2H); 2.27 (s,3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.6; 146.6; 137.9; 129.6; 129.3; 128.7; 126.1; 111.5; 57.6; 12.4
8	$(CH_2)_2OH$	Ethanol	203-4 (199-200 lit)[14]	68	^{1}H NMR (300 MHz, CD_3OD): δ 7.59 (d, *J* = 7,14 Hz, 1H); 6.38 (d, *J* = 7.14 Hz, 1H); 4,13-4.16 (m, 2H); 3.79–3.81 (m, 2H); 2.45 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 169.5; 145.9; 138.4; 132.0; 111.2; 60.1; 55.7; 11.0.

2.3 Synthesis of functionalized polyethylenimine succinylate (**PES**).

In a microwave reactor PEI (H_2O sol. 50%, 600-1.000 KDa, 2.5g) was dissolved in water (20 mL) and maltol **4** (0.5 g, 3.9 mmol) was added. The resulted solution was heated at 110°C for 1 h in a microwave apparatus (500-600W). After the TLC control (CH_2Cl_2/MeOH 9:1) showing the disappearance of maltol. the solution was transferred in a flask and succinic anhydride (2.0g, 20 mmol) and TEA (1.4 mL, 10 mmol) were added. The solution was stirred at room temperature overnight. 1M $NaOH_{aq}$ was then added until pH=11 and the solution was stirred for another 1 h. 10% HClaq was then added until pH = 5. The solution was purified by dialysis (dialysis sack MWCO 12000 Da) and finally lyophilized. Yield 1.1 g.

2.4 Synthesis of functionalized polyethylenimine acetylate (**PEA**).

In a microwave reactor PEI (600-1.000 KDa, 2.5g) was dissolved in water (20 mL) and maltol **4** (0.5 g , 3.9 mmol) was added. The resulted solution was heated at 110°C for 1 h in a microwave apparatus (500-600W). After the TLC control (CH_2Cl_2/MeOH 9:1) showing the disappearance of maltol, the solution was transferred in a flask and acetic anhydride (1.98 mL , 20 mmol) and TEA (1.4 mL, 10 mmol) were added. The solution was stirred at room temperature overnight. 1M $NaOH_{aq}$ was then added until pH=11 and the solution was stirred for another 1 h. 10% HCl_{aq} was added until pH = 5. The solution was purified by dialysis (dialysis sack MWCO 12000 Da) and finally lyophilized. Yield 1,2 g;

2.5 Synthesis of functionalized CMC (**fCMC**).

To a solution of CMC sodium salt (0.5 g) in water (50 mL), HOBT (0.8 g,5.9 mmol) and EDC (0.8g, 5.9 mmol) were added portionwise, until complete dissolution. Then 1-(3-aminopropyl)-3-hydroxy-2-methyl-pyridin4(1H)-one 5 (0.45 g, 2.4 mmol) was added and the solution was stirred overnight. The reaction mixture was then purified by dialysis (dialysis sack MWCO 12000 Da) and finally lyophilized. Yield 0.35g

2.6 Determination of Fe(III) complexation

A known mass of **PES** or **PEA** or **fCMC** was suspended in a 10 mM Fe^{+3} solution in deionized water at 25°C for 24 h. The remaining soluble (unbound) iron concentration was determined by atomic absorption spectroscopy and subtracted from the starting iron concentration. These values were used to calculate iron complexation per gram of iron binding polymers (Table 2).

Table 2. Calculated iron complexation per gram of polymer.

Polymer	Initial mmol of Fe^{+3}	Not complexed mmol of Fe^{+3}	complexed mmol Fe^{+3}	mmol Fe /1 g polymer
PES	9.99	2.71	7.28	9.71
PEA	9.99	0.53	9.46	9.99
fCMC	9.99	1.15	8.84	2.20

Chemical equilibria of iron ions in aqueous solutions are very complicated and the numerous studies carried out to identity the species formed and investigate their stability are controversial.[15] In fact, the speciation of iron depends on the nature of the complexes and on the environmental conditions. Generally, Fe(III) are not stable in the absence of any buffer causing the precipitation of $Fe(OH)_3$. In order to overvome this limitation, $FeCl_3$ was used to prepared ferric ions solutions. In fact, the acidity of $FeCl_3$ aqueous solutions, due to the formation of HCl in solution, guarantees the complete solubility of cations.

2.7 Determination of the complexation of Ca^{++}, Cu^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Mn^{++} , Cd++, Pb++

A known amount of **PES** or **PEA** or **fCMC** was suspended in 10 mM Ca^{+2} or Cu^{+2} or Ni^{+2} or Zn^{+2} or Mg^{+2} or Mn^{+2} or Cd^{+2} or Pb^{+2} solution in deionized water at 25°C for 24h. For each of them the remaining soluble metal concentration was determined by atomic absorption spectroscopy and subtracted from the starting metal concentration. These values were used to calculate each metal complexation. The results are showed in tables 3-5.

Table 3. Calculated metal complexation per gram of PES polymer

Metal	Initial mmol of M^{+2}	Not complexed mmol of M^{+2}	complexed mmol of M^{+2}	mmol Metal /g polymer

Ca	10.00	1.33	8.67	0.22
Zn	10.00	0.37	9.63	0.96
Cu	10.00	0.69	9.31	0.93
Ni	10.47	0.19	9.81	0.98
Pb	10.00	2.80	7.20	0.72
Cd	10.00	3.20	6.80	0.68

Table 4. Calculated metal complexation per gram of PEA polymer

Metal	mmol of M^{+2}	Not complexed mmol of M^{+2}	complexed mmol of M^{+2}	mmol Metal /g polymer
Ca	10.00	3.39	6.61	0.66
Zn	10.00	2.97	7.03	0.70
Cu	10.00	1.23	8.76	0.88
Ni	10.47	1.50	8.96	0.86
Pb	10.00	4.49	5.51	0.55
Cd	10.00	4.76	5.24	0.52

Table 5. Calculated metal complexation per gram of fCMC polymer

Metal	mmol of M^{+2}	Not complexed mmol of M^{+2}	complexed mmol of M^{+2}	mmol Metal /g polymer
Ca	10.71	1.87	8.84	2.03
Zn	10.00	2.30	7.70	1.92
Cu	10.00	2.52	7.48	1.87
Ni	10.47	2.06	8.40	1.97
Pb	10.00	4.22	5.78	1.45
Cd	10.00	3.70	6.30	1.57

2.8 Selectivity studies in simulated intestinal fluid.

Metal binding selectivities of **PES** or **PEA** or **fCMC** were determined in the presence of copper, zinc, cadmium, nickel, and calcium. A solution containing all these metal ions, each at a concentration of

0.4 mM, was prepared in a simulated intestinal fluid.[16] A predetermined amount of **PES** or **PEA** or **fCMC** was added into the solution and incubated at room temperature for 3 days. The concentration of each metal ion remaining in solution was determined by atomic absorption spectroscopy and subtracted from the starting respective metal concentration. These values were used to calculate the amount of each metal bound to funzionalized polymers (Table 6).

Table 6. Calculated metal complexation per gram of in simulated intestinal fluid

Metal	**PES** (mmol Metal /g polymer)	**PEA** (mmol Metal /g polymer)	**fCMC** (mmol Metal /g polymer)
Fe	9.30	9.90	2.47
Ca	0.90	0.89	2.25
Zn	0.98	0.92	2.22
Cu	0.95	0.94	2.36
Ni	0.81	0.83	2.05
Cd	0.47	0.54	1.26

2.9 Caco-2 cell monolayer integrity measurement

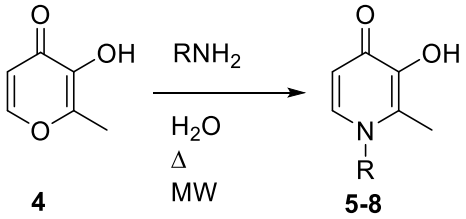
Caco-2 cells, obtained from INSERM (Paris) were routinely sub-cultured at low density (50%) and maintained at 37 °C in a 90% / 10% air / CO2 atmosphere in DMEM containing 25 mM glucose, 3.7 g/L $NaHCO_3$, 4 mM stable L-glutamine, 1% non-essential amino acids, 100 U/L penicillin, 100 µg/L streptomycin (complete medium), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, US). For differentiation, cells were seeded on polycarbonate filters, 12 mm diameter, 0.4 µm pore diameter (Transwell, Corning Inc., Lowell, MA, US) at a $3.5x10^5$ cells/cm^2 density in complete medium supplemented with 10% FBS in both AP and BL compartments for 2 days to allow the formation of a confluent cell monolayer. Starting from the third day after seeding, cells were transferred to complete medium in both compartments, supplemented with 10% FBS only in the BL compartment, and allowed to differentiate for 21 days with regular medium changes three times weekly.

Differentiated Caco-2 cells were treated with **PES** or **PEA** or **fCMC** samples at the final concentrations of 1.0 and 5.0 mg/mL for 15, 30, 120, 60, 180 min and 16 h, respectively. To determine the effects of the treatments on the permeability of intestinal tight junctions and the integrity of the cell monolayer in Caco-2 cells, Trans-Epithelial Electrical Resistance (TEER) was measured at 37 °C using the voltmeter apparatus Millicell (Millipore , Merck Group, Darmstadt, Germany) provided with Ag/AgCl electrodes, as previously described. TEER was expressed as $\Omega \cdot cm^2 = (\Omega \text{ cells} - \Omega \text{ filter}) A$, where Ω cells is the monolayer resistance, Ω filter is the resistance of the filter by itself and A is the filter area (cm^2).

Results and Discussion

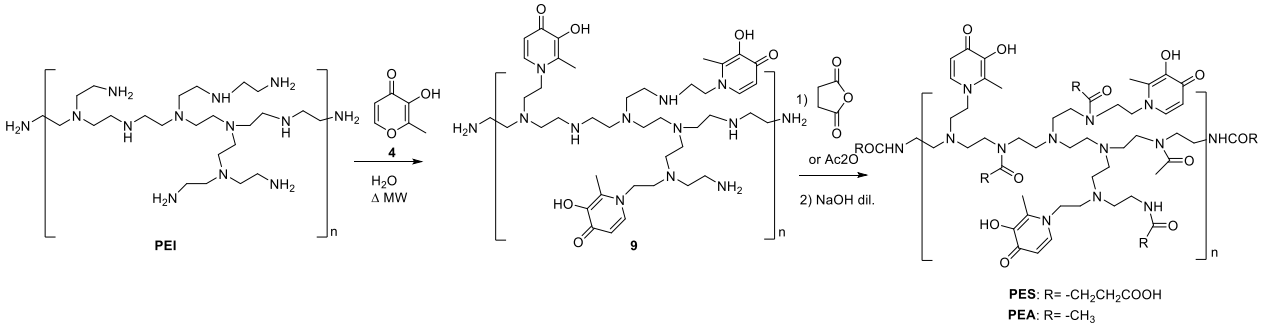
The 3-hydroxypyridin-4-ones (HPOs) are a class of compounds highly selective for Fe(III). Deferiprone is the lead compound and several HPO functionalized polymers have been proposed, although their synthesis is not so trivial.[17] Here, HPO has been incorporated into two chemically different polymeric structures, such as: branched Polyethylenimine (PEI), and Carboxymethyl cellulose (CMC). Branched PEI is a cationic polymer composed by repeated units containing primary, secondary and tertiary amino groups and two aliphatic ethyl spacers. The presence of the primary

amino groups could allow PEI to react with maltol leading to *N*-alkyl-hydroxypyridin-4-one PEI derivatives. The synthesis of *N*-alkyl-hydroxypyridin-4-one from *O*-benzyl protected maltol is known and widely used.[18] Moreover, the direct reaction of free maltol with amines at reflux has been also proposed but with poor yields.[19] In order to increase the yield, we investigated the possibility to apply microwave (MW) irradiation to the reaction (Scheme 1). The use of MW was indeed proposed for the preparation of deferiprone directly from maltol in 1994 with a 65% yield.[20]



Scheme 1: HPOs synthesis.

First, we studied the reaction on a simplified system, starting from maltol **4** in water and using different amine substrates (2 eq, Table 1). The MW irradiation was carried out at 110 °C for 2 h. In all cases the maltol completely converted to the products. The reaction was then performed on branched PEI (600-1000 Kda, 2.5g) that was reacted with maltol **4** under the same reaction conditions affording compound **9** (Scheme 2).



Scheme 2: PES and PEA synthesis

In order to avoid the presence of primary amino groups, they were then acylated using succinic anhydride or acetic anhydride. In both cases, the obtained derivatives were treated with diluted NaOH and, finally, the solutions were dyalized affording compounds **PES** and **PEA**, respectively.

As expected, both PES and PEA were found totally insoluble in the most common organic and aqueous solvents, as a consequence the materials were deeply characterized by FT-IR and the corresponding FT-IR spectra are reported in Figure 2.

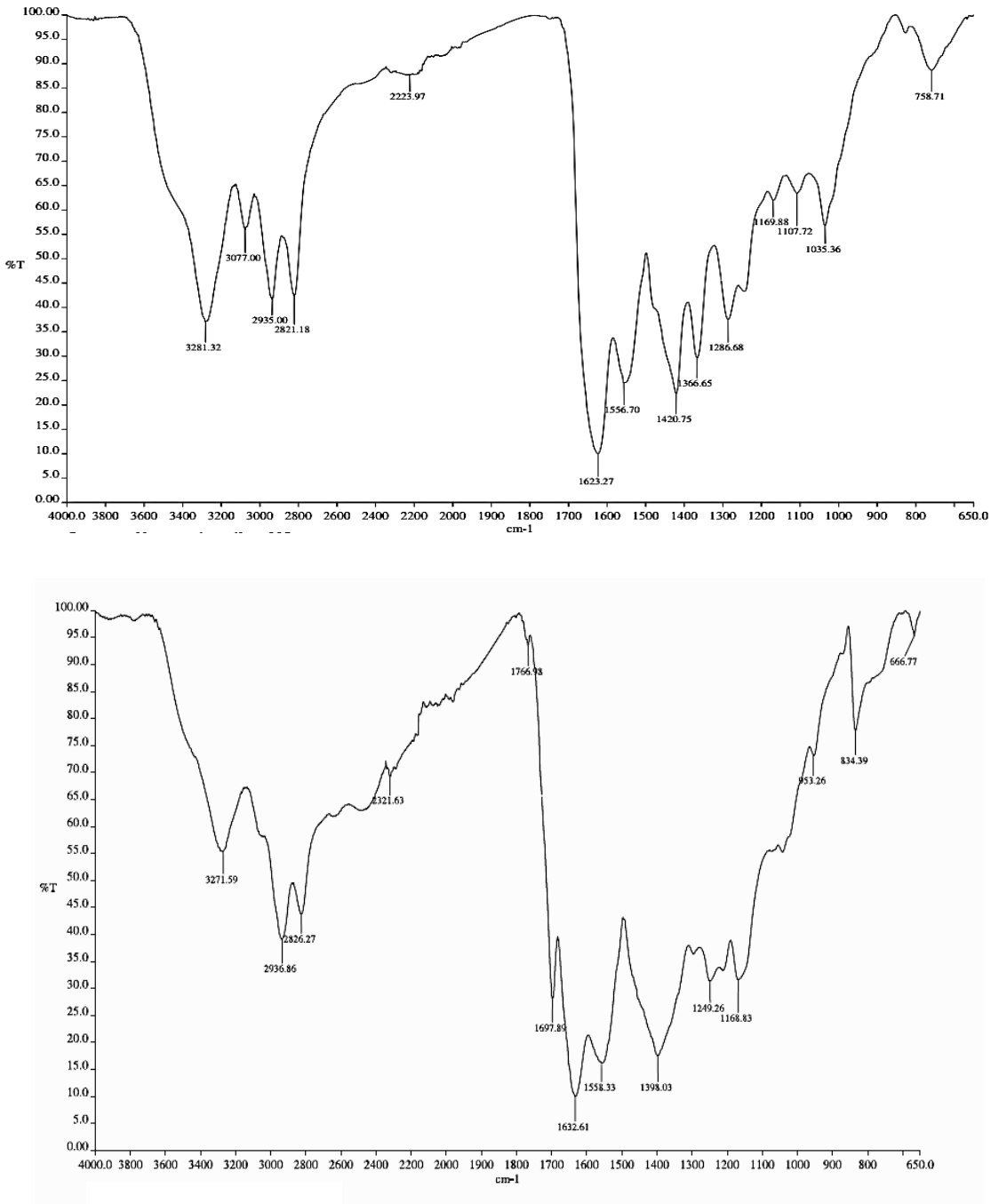
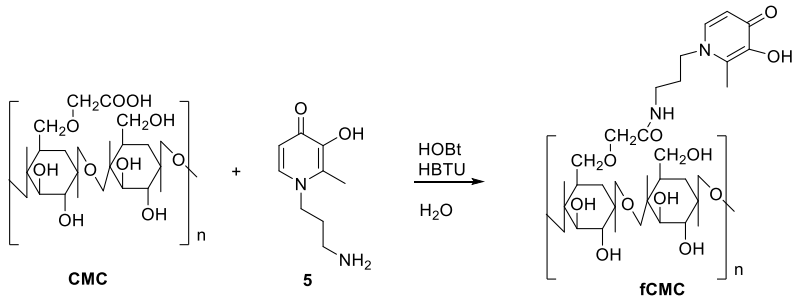


Figure 2: FT/IR spectra of **PES** (up) and **PEA** (down).

The **PES** spectrum showed the typical bands at 1697 (stretching of carboxylic acid), 1632 (stretching of amide I band) and 1558 (stretching of carbonyl group of conjugated keton) cm^{-1}. The band at 3271 cm^{-1} can be attributed to the -O-H stretching bond, whereas the bands at 2935 and 2821 cm-1 can be attributed to -C-H stretching bonds. The presence of the carboxylic group is ensured by the stretching of carbonyl at 1766 cm^{-1}. Finally, the band at 1398 cm^{-1} can be related to the C=C stretching. **PEA** spectrum showed similar absorption bands, except the characteristic band of carboxyl group that disappeared.

Being available aminopropyl functionalized compound **5**, we envisaged its exploitation for the functionalization of CMC, a cellulose derived polymer containing free carboxylic groups. The coupling reaction between CMC and **5** was performed in water, using HOBt and HBTU as coupling reagents (Scheme 3). After dialysis, functionalized **fCMC** was obtained and characterized by FT-IR technique, as reported in Figure 3.



Scheme 3: fCMC synthesis

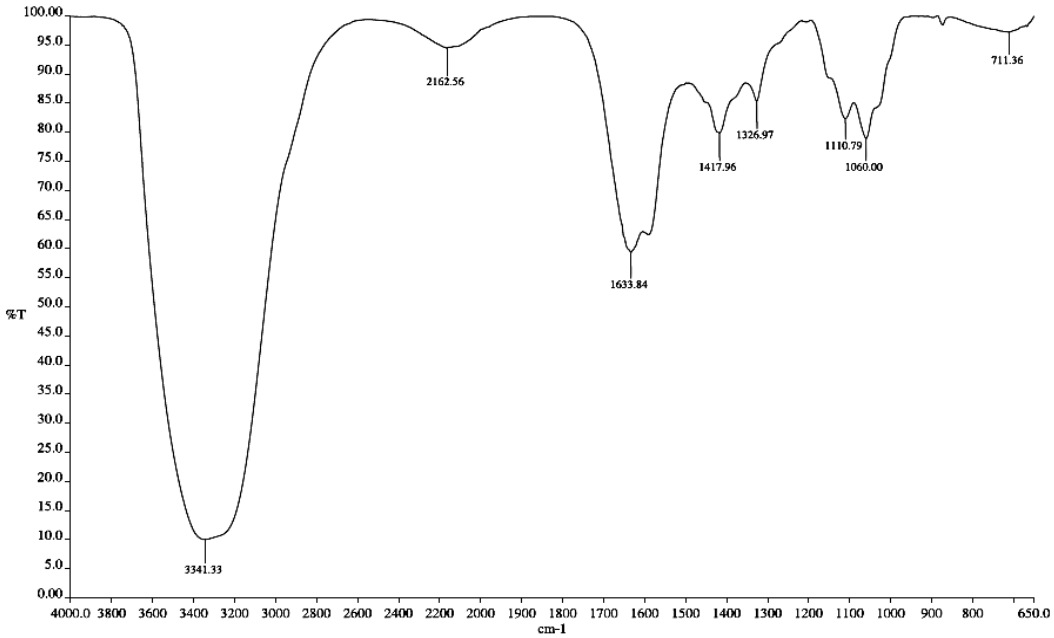


Figure 3: FT/IR spectrum of fCMC.

The presence of two strong absorption bands at 1635 and 1558 cm^{-1} confirmed the presence of amide group and conjugated keton. The broad absorption band at 3341 cm^{-1} is correlated to the stretching frequency of the –OH group, whereas the stretching vibration of C-H group was observed at 2930 cm^{-1}. The band at 1418 cm^{-1} and 1327 cm^{-1} are assigned to $–CH_2$ scissoring and –OH bending vibration, respectively and the band at 1060 cm^{-1} shows CH-O-CH_2 stretching.

The Iron binding capacity of these three polymers was evaluated using atomic absorption spectroscopy (AAS), allowing to determine the amount of loaded Fe(III) per gram of polymer as difference between the iron concentration in solution before and after the addition of polymers. All the functionalized polymers were incubated with Fe(III) solutions for 24 h and the obtained binding capacities are reported in Figure 4. In all case the polymers were found able to complex Fe(III), being functionalized PEIs the most efficient ones.

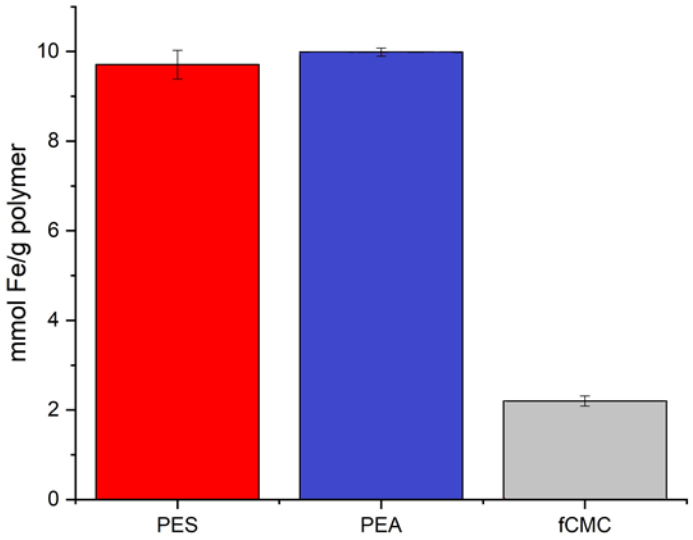


Figure 4: amount of loaded Fe(III) per gram of polymer.

The binding ability of the polymers toward other metal ions (such as copper, zinc, manganese, nickel, and calcium) was also investigated using AAS under the same operative conditions. It was found that the three polymers bind more efficiently Fe(III) with respect to the other ions (Tables 3-5). The specificity toward Fe(III) was also tested in simulated intestinal fluid.[16] As reported in Figure 5, **PES** and **PEA** polymers bound selectively Fe(III), while **fCMC** derivative was found less selective under these conditions.

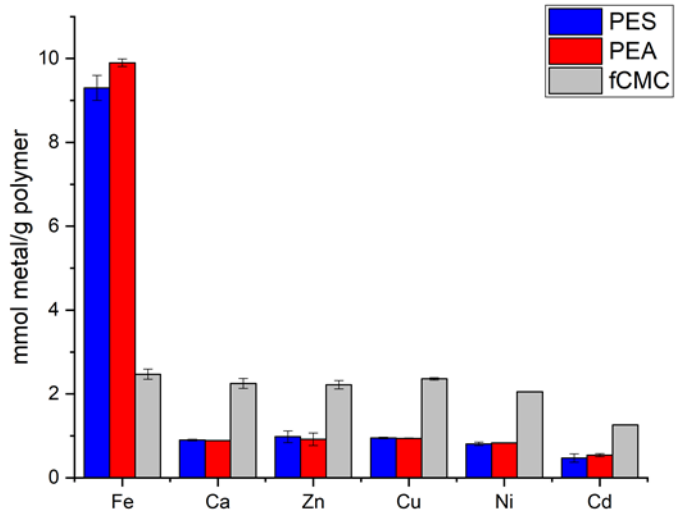


Figure 5: amount of loaded metals per gram of polymer.

In order to evaluate the effect of the polymers on enterocytes cells, Caco-2 cells monolayer was used as model. Indeed, upon differentiation, human intestinal Caco-2 cells display many of the physiological and morphological characteristics of mature intestinal enterocytes.[21] When differentiated on a filter insert (Transwell system), Caco-2 cells give rise to a polarized monolayer that separates two distinct compartments. The apical side (AP), which corresponds to the intestinal

lumen, and the basolateral compartment (BL), which corresponds to the interstitial space.[22] This cellular model was exploited to evaluate the potential toxicological effect of polymeric samples through the measurement of the TEER, which is a parameter of epithelial cell monolayer that reflects the functionality of the tight junctions.
In particular, PES, **PEA**, and **fCMC** samples (1.0 and 5.0 mg/mL) were incubated in the AP side of differentiated Caco-2 cells for 15, 30, 60, 120, 180 min and 16 h. Our results clearly indicate that no decrease in Trans-Epithelial Electrical Resistance (TEER) values was observed for tested samples vs control samples, suggesting that all the samples do not induce early sub-lethal cell toxicity and the integrity of the cell monolayer is not affected (Figure 6).

Moreover, migration from the apical to the basolateral side was not observed, hence these non-absorbable polymers are suited for oral-enteral iron sequestration.

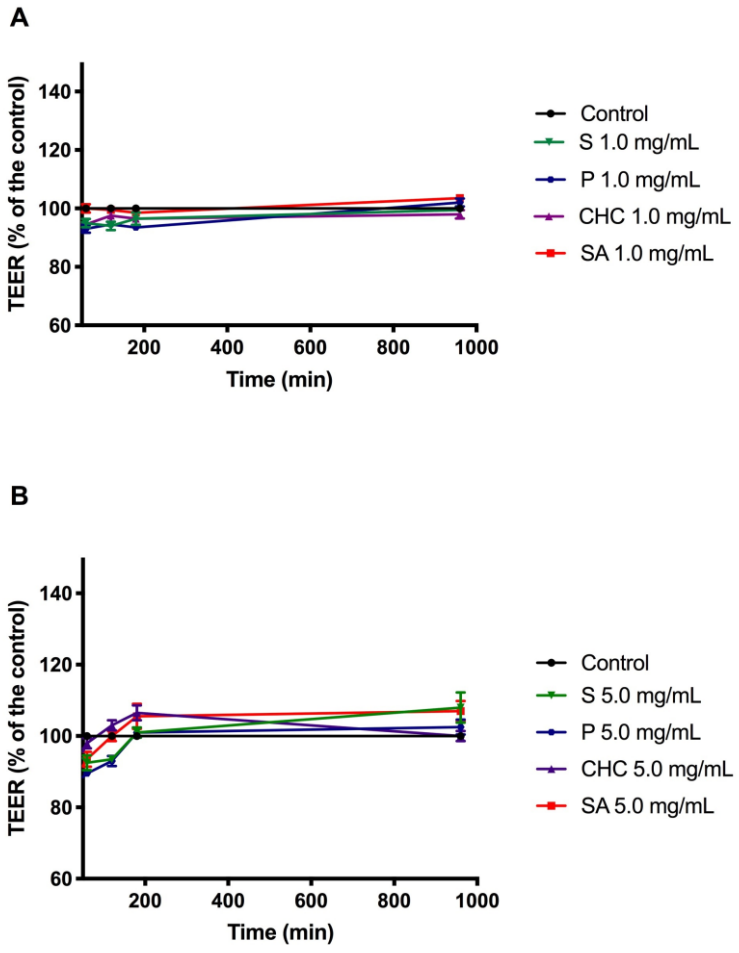


Figure 6: Effect of **PES**, **PEA** and **fCMC** on TEER value modulationat 1.0 (A) and 5.0 (B) mg/mL, respectively, as function of the time. Lines represent the average of 3 independent experiments in triplicate

Conclusions

In conclusion, we synthesized three different IBPs containing the 3-hydroxypyridin-4-one Fe(III) chelating functional group linked to PEI and CMC polymers. We demonstrated that each polymer is able to bind Fe(III), being functionalized PEI Fe(III) selective in the presence of other metal ions and in simulated intestinal fluid. Experiments carried out on Caco-2 monolayer showed that the polymers

are not toxic without altering the integrity of intestinal enterocytes. Therefore, functionalized PEIs represent promising compounds in the development on non-absorbable IBPs. In particular, the enacted intestinal Fe-starvation are conceived as candidate in a) treatment of oral iron side-effects after IDA therapy; b) prophylactic regimen in dysbiosis with manifested recurrent diarrhea, post-antibiotic enteritis, and enteral infections due to iron-avid pathogens; c) maintenance therapy in quiescent or mild IBD and supportive device in pharmacologic IBD protocols; d) preventive regimen in subjects predisposed to CRC, i.e. processed or high meat consumers, and under therapy of oral iron for IDA, or treatment after onco-therapy or surgery to decrease CRC relapse.

Declaration of Competing Interest

Carlo A Ghisalberti owns share in Tixupharma. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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