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# **THE CHELATION OF COLONIC LUMINAL IRON BY A UNIQUE SODIUM ALGINATE FOR THE IMPROVEMENT OF GASTROINTESTINAL HEALTH**

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**Abbreviations:** **IBD** (Inflammatory Bowel Disease), **ROS** (Reactive Oxygen Species), **G** (Guluronic acid), **M** (Mannuronic acid), **TfR1** (Transferrin Receptor 1), **AUC** (analytical ultracentrifugation), **ADPs** (Alginate Degrade Products), **AlgE1** (Alginate Epimerase 1) and **EpLD** (Epimerised Manuacol LD).

**Key Words:** Iron; Alginate; Chelation; Intestinal; Absorption.

## ABSTRACT

**Scope:** Iron is an essential nutrient. However, in animal models, excess unabsorbed dietary iron residing within the colonic lumen has been shown to exacerbate inflammatory bowel disease and intestinal cancer. Therefore the aims of this study were to screen a panel of alginates to identify a therapeutic that can chelate this pool of iron and thus be beneficial for intestinal health.

**Methods and results:** Using several *in vitro* intestinal models it is evident that only one alginate (Manuacol LD) of the panel tested was able to inhibit intracellular iron accumulation as assessed by iron mediated ferritin induction, transferrin receptor expression, intracellular <sup>59</sup>Fe concentrations and by measuring iron flux across a Caco-2 monolayer. Additionally, Manuacol LD suppressed iron absorption in mice which was associated with increased faecal iron levels indicating iron chelation within the gastrointestinal tract. Furthermore, the bioactivity of Manuacol LD was found to be highly dependent on both its molecular weight and its unique compositional sequence.

**Conclusion:** Manuacol LD could be useful for the chelation of this detrimental pool of unabsorbed iron and it could be fortified in foods to enhance intestinal health.

## INTRODUCTION

Iron is an essential nutrient with many cellular functions reliant upon iron-catalysed processes such as DNA synthesis and ATP generation.[1, 2] Total body iron levels in adult males is between 3000 – 4000 mg, with a daily nutritional need for iron of 20 mg which is mostly required for erythropoiesis.[3] This daily requirement of iron is ingested from the diet; however it is known that only 0.7 – 22.9 % of ingested non-heme iron is absorbed within the small bowel.[4] As a consequence the remaining dietary iron resides within the large bowel for hours to days. Recent reports have concluded that this 'luminal iron' (iron present within the lumen of the colon) has a detrimental effect on intestinal health.[5, 6] Most notably two recent murine studies have shown that removal of dietary iron from models of inflammatory bowel disease (IBD) and intestinal cancer resulted in a suppression of disease phenotype, whilst consumption of excess dietary iron exacerbated the conditions.[5, 6] How this excess iron is mediating these effects is unknown, however it could be via oncogenic signalling (the Wnt signalling pathway),[7] the generation of reactive oxygen species (ROS),[8-10] or through the modulation of the intestinal microbiome. [6, 11]

Iron chelation represents a platform for therapeutic intervention, whereby ligated iron would be neutralised and unable to partake in any toxicity-related processes; this has indeed been demonstrated in the context of chelation of excess systemic iron, where the use of iron chelators have been shown to have beneficial effects.[12] However, with respect to luminal iron a therapeutic agent must not demonstrate systemic iron binding and iron chelation must solely take place within the gastrointestinal tract. As such, a compound that demonstrates iron binding potential whilst having a limited bioavailability needs to be identified. Sodium alginates are a fibre found throughout many foods which demonstrate these physico-chemical properties.[13, 14]

Alginates sourced from algae are formed of unbranched, 1-4 linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G). The arrangement of G and M residues along the polymeric backbone sequence is ordered being heteropolymeric (e.g. GMGMGM) or homopolymeric (e.g. MMMGGG).[15] The physical properties of alginate can differ depending on their molecular weight and GM sequence; these structural differences give rise to a plethora of possible structural compositions. How these compositional differences may alter the biochemical actions of alginates is unknown, and in particular how they influence iron binding *in vitro* and *in vivo* is not known. As such, the impact of a range of alginates (representing different chemical compositions) on *in vitro* and *in vivo* cellular iron metabolism was assessed. Further to this, the physico-chemical properties of alginate which are crucial for its effects on cellular iron metabolism were examined to reveal the alginate chemical composition required for luminal iron chelation. Results from these experiments identify an alginate which is nutritionally beneficial and likely to be useful in enhancing intestinal health.

## **MATERIALS AND METHODS**

### **Cell culture**

RKO cells were grown in growth medium which consisted of Dulbecco's Modified Eagles Medium (DMEM) supplemented with foetal calf serum (FCS) (10 % v/v), penicillin (100 U ml<sup>-1</sup>) and streptomycin (0.1 mg mL<sup>-1</sup>). Caco-2 cells were grown using a similar growth medium which was supplemented with non-essential amino acids (1 % v/v). Both cell lines were purchased from the American Type Culture Collection (ATCC).

In experiments with iron co-incubation, a standard protocol was employed as previously described.[13] Throughout all experiments the form of iron used was FeSO<sub>4</sub>. To create alginate stimulation media, alginate (2 % w/v in DI H<sub>2</sub>O) was mixed with growth medium with or without iron (FeSO<sub>4</sub>·7H<sub>2</sub>O, 100 μM) to create a resultant 0.3 % alginate medium, with or without iron supplementation. These stimulation media were cultured with cells for 24 hours. After this time period, media was removed, cells washed with PBS and lysed in RIPA lysis buffer (1% 4-Nonylphenyl poly(ethylene glycol), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (w/v) in DI H<sub>2</sub>O).

### **Caco-2 monolayer**

Caco-2 cells were seeded into pre-treated collagen-coated 6-well transwell inserts at a concentration of 4 x 10<sup>5</sup> cells mL<sup>-1</sup>. Cells were grown for 20 days post confluency. Prior to culture with iron and/or alginate, cell medium was changed to FCS-free Minimum Essential Medium (MEM) as previously reported.[16] Subsequently, cells were co-cultured with iron (FeSO<sub>4</sub>·7H<sub>2</sub>O, 100 μM) and/ or alginate (0.3 %) as described above. To create <sup>59</sup>Fe iron media, a stock solution of iron (FeSO<sub>4</sub>·7H<sub>2</sub>O (108.9 mg, 10 mM) and sodium ascorbate (396 mg, 500 mM)) into DI H<sub>2</sub>O (40 mL) was spiked with <sup>59</sup>FeCl<sub>3</sub> to reach a radiation concentration of 10,000 counts per minute (CPM) per well. This stock was diluted into the media to create the 100 μM FeSO<sub>4</sub>·7H<sub>2</sub>O as detailed above. To the apical chamber, FCS-free MEM with <sup>59</sup>Fe

spiked iron with or without alginate (2 mL) was added. At 0.5, 4 and 24 hour time points, samples were removed from the apical chamber. After 24 hours, media was removed, the cells washed with Versene (0.2 g L<sup>-1</sup> EDTA in PBS) and lysed in RIPA buffer. Samples collected were assessed for iron concentration using scintillation counting.

### **Western blotting**

Cells were incubated in iron (FeSO<sub>4</sub>·7H<sub>2</sub>O, 100 μM) and or alginate (0.3 % w/v in DI H<sub>2</sub>O) or D-glucuronic acid (0.3 % w/v in DI H<sub>2</sub>O) supplemented growth media for 24 hours prior to lysis in RIPA buffer. Western blotting was performed as previously described, with monoclonal antibodies to ferritin (1:5000, Abcam, Rabbit AB69090), β-actin (1:5000, Abcam, Mouse AB8226) and TfR1 (1:1000, Invitrogen, H68.4).[13] All blots were subject to densitometry analysis using ImageJ analysing software.

### ***In vivo* <sup>59</sup>Fe experiments**

All *in vivo* experiments were carried out under Home Office approved conditions and Animal care and the regulation of scientific procedures met the criteria laid down by the United Kingdom Animals (Scientific Procedures) Act 1986. Male, 6 week year old, CD1 mice (Charles Rivers, UK) in groups of 4-8 were used in <sup>59</sup>Fe absorption experiments. All mice were starved 12 hours prior to gavage and post administration mice were given access to water and food *ad libitum*.

A <sup>59</sup>Fe spiked iron gavage solution was prepared by dilution of a Fe(II) stock solution (FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 mM in 0.1 M HCl) into HEPES, a physiological buffer devoid of divalent metal cations (HEPES, 16 mM pH = 7.4, NaCl 125 mM) to a concentration of 250 μM Fe(II). Administration of alginate solutions (8 % w/v in DI H<sub>2</sub>O, 100 μL) to half of the mice was performed immediately after gavage of the prepared radio-labelled iron (100 μL), whereas the other half received an iron only gavage and no alginate. All mice were housed in metabolic

chambers for 48 hours prior to culling to allow the collection of faecal samples. After 48 hours, mice were culled and dissected. Intestinal sections (duodenum, jejunum, ileum and colon) were flushed once with a sodium chloride solution (ca. 15 mL, 0.15 M) and detection of iron concentrations was performed within these intestinal sections as previously reported.[17] Carcass CPM measurements were performed on a whole body animal counter (LIVE-1, Technical Associates, Canoga Park, CA).

### **Heat degradation and viscosity measurements**

Aqueous Manurol DH aliquots (10 mL) were heated at 100 °C for set time points. All viscosity measurements were performed at 25 °C and alginate concentrations that were used were within the kinematic range of the viscometer (usually from 0.1 – 1 mg mL<sup>-1</sup>). Viscosities were measured on a Cannon-Ubbelohde glass viscometer (Cannon instruments), size 50, with a kinematic viscosity range of 0.8 – 4 mm<sup>2</sup> s<sup>-1</sup>.

### **Analytical ultracentrifugation**

Sedimentation velocity and sedimentation equilibrium experiments were performed on a Beckman Optima XL-I analytical ultracentrifuge (AUC) equipped with Rayleigh Interference Optics and a 30 mW laser wavelength  $\lambda = 675$  nm as previously reported.[18]

### **AlgE1 Plasmid extraction and enzyme production**

Agar blocks inoculated with JM 109 containing plasmid pHH1 encoding alginate epimerase enzyme 1 (AlgE1) was kindly donated from Helga Ertesvåg (Norwegian University of Sciences and Technology). AlgE1 production was performed according to the previously published protocol.[19]

### **Assessing for activity of AlgE1 and preparation of Epimerised LD**

To screen the purified enzyme fractions for epimerisation activity, aqueous Manurol LD (0.1%, (w/v)) in DI H<sub>2</sub>O was prepared. Manurol LD solution (2 mL) was mixed with the enzyme

fractions (1 mL) and MOPS buffer (80 mM, 1 mL) supplemented with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (4 mM). The resultant mixture was incubated at 37 °C for 2 hours. To quench the epimerisation, EDTA (50 mM) was added and the subsequent mixtures were extensively dialysed against DI  $\text{H}_2\text{O}$  at 4 °C. The resultant solutions were used directly in the circular dichroism (CD) spectrometer. CD measurements were recorded on a Jasco J-810 CD spectropolarimeter using a 1 cm path length, blackened, quartz cell. The selected active fraction was used to prepare Epimerised Manucol LD (EpLD) for cell culture experiments. Aqueous Manucol LD (0.2 %, w/v) was mixed with MOPS buffer (180 mM) supplemented with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (40 mM) and the active enzyme (20 mL). The resultant solution was agitated at 37 °C for 24 hours, before enzyme deactivation was initiated with the addition of EDTA (0.5 M). The mixture was then extensively dialysed at 4 °C. The subsequent alginate product was pH adjusted (pH = 7.4) and concentrated *in vacuo*.

### **High resolution NMR**

$^1\text{H}$  NMR spectra were acquired on a Bruker Avance III 600 MHz instrument equipped with a 5 mm TCI Cryoprobe. The residual solvent resonance was further suppressed using a NOESY presto pulse sequence. A total of 32 transients and 16 steady state scans were acquired with 16384 complex data points. The spectral width was set to 7184 Hz and the sample temperature to 340 K. The free induction decays were multiplied with a 0.3 Hz broadening exponential window function and zero filled to 32768 real data points prior to Fourier transformation. The spectra were then manually phase and baseline corrected, using a spline baseline correction. Signals for the anomeric protons of the different alginate constituents were fitted to lorentzian lines. All data processing and analysis was performed using the matlab based MetaboLab software package.[20]

### **Statistical analysis**

All experiments were performed at least in triplicate unless otherwise stated. Data was processed using Microsoft excel and results are presented as means with standard errors of the mean (+/- SEM). The single-factor analysis of variance (ANOVA, Analysis ToolPak,

Microsoft Corporation) was used to determine significant differences between the means of three or more independent (unrelated) groups. The unpaired t-test was used to statistically compare mean values between two unrelated data sets within a group. A level of significance was set to  $p < 0.05$  for both tests of significance.

## RESULTS

### **Manucol LD demonstrates iron chelation *in vitro***

To screen the iron binding potential of the range of alginates used in this study, RKO cells were challenged with iron (100  $\mu\text{M}$ ) in the presence or absence of sodium alginates (Manucol LD (LD), Manucol DH (DH), Manugel GHB (GHB), LFR5/60 (LFR), KELTONE (KEL), PROTANAL RF6650 (RF) and PROTSEA AFH (AFH), 0.3 % w/v) for 24 hours and cells were then subsequently assayed for ferritin expression; a surrogate marker for cellular iron levels (Figure 1A). As expected treating cells with iron supplemented media alone induced ferritin expression ( $p < 0.05$ ). Only one alginate (Manucol LD) was able to significantly decrease the iron induced ferritin response by 60 % ( $p < 0.05$ ). Similarly, culture of RKO cells at lower concentrations of iron (1 and 10  $\mu\text{M}$ ) in the presence or absence of alginates (LD, DH, GHB, LFR, KEL, RF and AFH, 0.3 % w/v) demonstrated that Manucol LD was the only alginate to significantly reduce ferritin expression by 70, 88 and 68 % at 1, 10 and 100  $\mu\text{M}$  concentrations of iron respectively compared to the iron only control ( $p < 0.05$ ) (Figure 1B). DH, GHB and LFR only inhibited ferritin expression at 10  $\mu\text{M}$  iron by 54, 47 and 92 % respectively ( $p < 0.05$ ) (Supporting Information Figure S1). Manucol LD was the only alginate to demonstrate bioactivity at all concentrations of iron.

In order to fully verify the iron chelation ability of Manucol LD *in vitro*, RKO cells were challenged with iron (100  $\mu\text{M}$ ) with or without Manucol LD (0.3 % w/v) for 24 hours before being assessed for direct intracellular iron concentrations (Figure 1C) and Transferrin Receptor 1 (TfR1) expression (Figure 1D). RKO cells treated with iron resulted in cellular iron loading (ca. 50 nM total cellular iron); the basal levels of iron within the control group were not measureable by this assay. It was found that Manucol LD significantly decreased cellular iron loading by 62 % ( $p < 0.05$ ) (Figure 1C). This was associated with a statistical increase in TfR1 expression by ca. 50 % ( $p < 0.05$ ) compared to iron alone, indicating that Manucol LD is

binding the supplemented iron present in the media hindering its intracellular uptake (Figure 1D).

### **Manucol LD demonstrates iron chelation in an *in vitro* model of the intestinal lumen**

Using radiolabelled iron-spiked cell culture media, Manucol LD significantly decreased intracellular iron concentrations by 70 % ( $p < 0.05$ ) within the Caco-2 monolayer compared to the iron only control (Figure 2A). By assessing the levels of iron within the apical chamber during the experiment it could be determined that iron was retained within this compartment (Figure 2B). Co-incubation with Manucol LD at both 4 and 24 hours inhibited cellular iron uptake by the Caco-2 cells by 60 % ( $p < 0.05$ ) compared to the iron only control from the apical compartment. These results validate the observation that Manucol LD is binding iron in the media thus preventing its cellular internalisation.

### **Manucol LD demonstrates iron chelation within the gastrointestinal tract *in vivo***

Administration of Manucol LD resulted in significantly decreased carcass iron concentrations by 71 % ( $p < 0.005$ ) compared to an iron only cohort (Figure 3A). This equates to only 13 % of the total iron administered being absorbed when Manucol LD was present compared to 46 % when it was absent (Supporting Information Figure S2). In addition, there was a concomitant increase in faecal iron concentrations of 45 % ( $p < 0.05$ ) in mice administered Manucol LD compared to mice administered iron alone (Figure 3B). To fully verify the iron chelation potential of Manucol LD throughout the gastrointestinal tract, the stomach, duodenum and colon were assayed for iron concentration and it was found that in all gastrointestinal tissues there were statistically decreased iron concentrations when mice were administered Manucol LD compared to iron alone (Figure 3C). Specifically, a significant decrease of 53, 60 and 52 % ( $p < 0.05$ ) in the stomach, duodenum and colon was found respectively. Moreover, the iron concentrations significantly increases from the stomach to

the duodenum to the colon (stomach → duodenum 73% increase, stomach → colon 83 % increase ( $p < 0.05$ )). All mice received the same concentration of  $^{59}\text{Fe}$  radioactive gavage (Figure 3D).

### **Chemical characterisation of alginates**

To establish why Manucol LD demonstrated bioactivity and the other alginates did not, chemical characterisation of the alginate series was performed. Both assessment of molecular weight by analytical ultracentrifugation (AUC) (Supporting Information Figure S3) and G:M composition by nuclear magnetic resonance (NMR) (data not shown) was performed.[21, 22] A summary of these results are described in Table 1. Manucol LD was found to have a molecular weight distribution of 145 kDa. Manucol LD was also found to have a G:M ratio of 38:62, however, this composition was not unique to Manucol LD as Manucol DH had a similar G:M ratio of 40:60.

### **Alginate iron chelation is molecular weight dependent**

Since Manucol DH and Manucol LD shared similar G:M composition homology, yet Manucol DH was found to have a higher molecular weight than Manucol LD (cf. 170 vs 145 kDa), to determine if the difference in bioactivity was due to the molecular weight of Manucol DH, Manucol DH was subject to heat degradation to produce smaller molecular weight average alginate degrade products (ADPs) as previously described.[23] The relative viscosity decreases as expected with longer heating times, and the mean values for intrinsic viscosity obtained can be calibrated against heating time (Figure 4A). The resultant ADPs (0.3 % w/v) were subsequently co-cultured in the presence or absence of iron (100  $\mu\text{M}$ ) *in vitro* for 24 hours to examine their effects on iron induced ferritin expression (Figure 4B). Co-culturing RKO cells with native Manucol DH does not demonstrate any iron chelation effects as described previously (Figure 1A). Heat degradation of Manucol DH for 20 and 40 mins and subsequent co-culture of these ADPs on RKO cells also reveals no iron binding ability. However, heat degradation for 80 and 160 min statistically reduced iron-mediated ferritin

expression by ca. 50 % ( $p < 0.05$ ), but not to the extent of Manucol LD (Figure 4B). Further degradation (250 min) resulted in a loss of this bioactivity. As a further control, the effect of the monomeric-acid unit on cellular iron metabolism was assessed. D-glucuronic acid (DGA) (0.3 % w/v) was co-cultured alongside RKO cells in the presence or absence of iron (100  $\mu$ M). There was no statistical reduction in iron induced ferritin expression or  $^{59}\text{Fe}$  levels when cells were cultured alongside DGA compared to iron only (Figure 4C & D).

### **Alginate iron chelation is composition dependent**

Manucol LD and Manucol DH have similar G:M chemical compositions but this does not preclude differences in GM sequence structure. To examine this they were both subject to high resolution NMR to allow calculation of their respective monad (G/M), diad (GM/GG/MM) or triad (GMG/GGM/MGM/MMG) concentrations.[24] NMR spectra for Manucol LD and Manucol DH were acquired and analysed using the correlations described and transformed free-induction decays were fitted to lorentzian curves to allow calculation of the specific monads, diads and triads (Figures 5A and 5B) according to a standard protocol.[24] Calculation of the major differences between Manucol LD and Manucol DH revealed that Manucol LD contained 12 % more MM diads than Manucol DH. Similarly, Manucol DH contained 8 % more GM diads than Manucol LD.

To interrogate the dependence on G:M composition and sequence structure, an alginate epimerase enzyme (AlgE1) was produced and utilised for the M $\rightarrow$ G conversion of Manucol LD. Epimerisation of native Manucol LD by AlgE1 resulted in an approximate two-fold increase in guluronate residues on epimerised Manucol LD (EpLD) resulting in a new G:M ratio of 77:23; the highest G-unit concentration alginate out of the series as assessed by circular dichroism spectroscopy (Figure 5C). There was also very little absorption present at  $\lambda = 280$  nm on the UV-Vis indicating negligible AlgE1 protein contaminant. To examine the effects of epimerisation of Manucol LD on iron induced ferritin expression, RKO cells were co-cultured in the presence or absence of Manucol LD or EpLD with or without iron for 24 hours

before ferritin expression was examined (Figure 5D). Ferritin expression in RKO cells treated with iron was significantly higher than that of control media only, as expected. Manurol LD statistically diminished ferritin expression by 57 % compared to iron only control (cf. 60 % in previous studies) ( $p < 0.005$ ). EpLD did not reduce ferritin expression compared to the iron only control, and expression was statistically increased by 63 % compared to Manurol LD treatment ( $p < 0.005$ ) (Figure 5D).

## DISCUSSION

The detrimental effect of excess iron within the colon has previously been reported.[5, 6, 25, 26] The ingestion of high amounts of red and processed meats, foods that contain high levels of iron, have also been implicated in gastrointestinal disease.[27, 28] Exactly how iron is mediating disease progression within the large bowel is currently unknown. Despite this, in a range of conditions associated with iron excess, there is evidence that iron chelation is therapeutically beneficial.[29] However, in the context of gastrointestinal disease it is specifically an excess of *luminal iron* within the large bowel that is detrimental and the chelation of this pool of iron presents itself as a potential therapeutic platform to improve intestinal health.

In order to selectively chelate iron within the lumen of the colon the therapeutic compound must not be absorbable such that it reaches the large bowel and binds excess iron. Non-digestible fibres have been demonstrated to improve gastrointestinal health,[30] yet whether these effects are attributed to their iron binding properties is unknown. Sodium alginates which are fibres found within the human diet have previously been demonstrated to be both non-absorbable and bind iron.[13, 14] The possible range of alginate compositions is huge due to the diversity in polymer length and G:M chemical composition.[31, 32] How these compositional differences affect iron binding potential and subsequent *in vitro* and *in vivo* cellular iron modulation is unknown, with previously published reports being inconsistent in their findings.[14, 33-35] This inconsistency is likely due to the fact that chemically different alginates were used throughout these studies, and data presented in this study demonstrates the dependence of iron chelation bioactivity on alginate chemical composition.

A range of alginates with different molecular weights and G:M compositions were primarily screened to assess their *in vitro* iron chelation potential. This initial assessment identified only one alginate that was able to modulate iron metabolism *in vitro*. Manucol LD decreased

ferritin expression by 60 % (Figure 1A and B), decreased total cellular iron by 62 % (Figure 1C), restored TfR1 expression to control levels (Figure 1D) and decreased absorption of iron in Caco-2 cells by 70 % (Figure 2 A and B); this was the only alginate to have consistent iron chelation properties (all statistically significant to  $p < 0.05$ ). *In vivo*, Manucol LD was able to suppress iron absorption by 71 % and increased the iron content found in faecal samples by 45 % ( $p < 0.05$ ) (Figure 3). These two results are pivotal in demonstrating the mechanism of iron binding by Manucol LD within the gastrointestinal tract, where it can be inferred that Manucol LD is binding iron and keeping it chelated until defecation. Where the iron is interacting with Manucol LD within the gastrointestinal tract remains unclear, however, as alginates are insoluble in low pH environments and form insoluble capsules, it is likely that iron chelation would not take place until Manucol LD reached more basic, distal regions of the gut.[36] Interestingly, the concentrations of iron within the mucosa of gastrointestinal tissues increases from the small to the large bowel (stomach<duodenum<colon). This is likely to be a consequence of either i) more effective iron transport across the brush border in the duodenum than within the colon, ii) the increased length of exposure to iron in the digesta within the colon, or iii) a combination of both. This finding is supported by studies which have demonstrated that the colon is able to import iron but there is a lack of regulated iron efflux.[37] Thus unsurprisingly, Manucol LD had its greatest iron chelation effects within the colon decreasing iron absorption by 56 % ( $p < 0.05$ ).

Manucol LD has iron chelation effects both *in vivo* and *in vitro* however, what remains unclear is what the redox state and likely species of iron that alginate is chelating to within these experimental conditions. Since sodium ascorbate was included within the growth media in cell culture experiments it is likely that the supplemented ferrous iron will remain in the 'free' ferrous form ('free' referring to its availability to chelation by competing ligands) which would suggest that Manucol LD is certainly binding to free ferrous iron. In addition previous reports have indicated that alginate will also bind ferric iron.[13] It can be inferred that Manucol LD is likely to have iron chelation ability towards ferrous and ferric ions, yet this does not rule out

the possibility that iron could be forming nanoparticulate species within the gastro-intestinal tract,[38] which alginates have also been documented to stabilise.[39] If indeed iron in its 'free' form is present within the colon then these data suggest that Manucol LD is able to bind such a form of iron and possibly nanoparticulate forms based on previously published reports.[13] However, the species of iron present within the colon is currently unknown and could be in its 'free' state, bound to other dietary or endogenous intestinal compounds or even as particulate mineralised species.[38, 40, 41]

To interrogate the chemical characteristics of alginate required for iron chelation and subsequent modulation of cellular iron metabolism, Manucol LD and Manucol DH (the latter an alginate with similar G:M composition but different polymer length) were subject to structural modification by alginate degradation (through heating) and M→G unit conversion (by exposure to alginate epimerase AlgE1). Determination of the heating time required to reduce molecular weight by specific amount allowed the calibration of intrinsic viscosity change against heating time (Figure 4A). Heat degradation of Manucol DH at 100 °C decreased with heating time as previously reported.[42] It was estimated that heating Manucol DH for ca. 3 hours would provide an intrinsic viscosity value of 1200 mL g<sup>-1</sup>, which would give a molecular weight similar to that of Manucol LD (145 kDa). As such, heating Manucol DH for 180 min produced a shorter chain alginate (analogous to Manucol LD) that, when co-cultured *in vitro* with iron decreased iron induced ferritin expression by 50 % ( $p < 0.05$ ) (Figure 4B). As a control, Manucol DH was heated for longer periods of time (250 min) to produce shorter molecular weight products. Subsequent co-culture of these ADPs resulted in diminished iron chelation ability to that observed with the 180 min ADPs as ferritin levels were not significantly different to the iron only control. As a further control, RKO cells were co-cultured with iron in the presence of D-glucuronic acid (DGA) to examine if the individual monomeric acid-unit was able to modulate cellular iron metabolism; DGA also had no significant effects on ferritin expression or intracellular <sup>59</sup>Fe concentration. These results demonstrate the importance of polymer length on iron binding ability, and it has recently been reported how the polymeric

nature of alginate orchestrates iron chelation.[13] Thus in summary an alginate molecular weight of approximately 145 kDa is required for maximal iron chelation bioactivity.

It is plausible that Manuacol LD has a tertiary and secondary structure that forms an iron binding pocket or cavity; this structure is formed by the specific MG sequence of the alginate. The iron binding site acts as a nucleation site for iron deposition and such mechanisms have been previously reported for other biopolymers.[39] If this were the case, then alteration of the G:M ratio and thus GM sequence on Manuacol LD would disrupt the formation of this binding site. Indeed, data presented in this study has demonstrated this. Alginate Epimerase 1 was utilised to convert Manuacol LD (G:M 38:62) to EpLD (G:M 77:23). Subsequent co-culture of EpLD in the presence of iron did not reduce ferritin expression as demonstrated for native Manuacol LD. Such results demonstrate the importance of a specific alginate G:M ratio and GM sequence in iron chelation bioactivity. To fully confirm the uniqueness of Manuacol LD, high resolution NMR spectroscopy showed that Manuacol DH and Manuacol LD (two alginates with similar G:M ratio) do indeed have different GM sequence homologies, where differences in MM and GM (or MG) diad frequencies are most apparent.

In summary, data presented here identifies a unique alginate, with a specific chemical composition and polymeric length that demonstrates optimal iron chelation properties both *in vitro* and *in vivo*. This data not only explains why inconsistencies have previously been reported on alginate modulation of cellular iron metabolism but also detail the required chemical characteristics of an alginate for iron chelation bioactivity. Importantly, if Manuacol LD is to be used to chelate excess colonic luminal iron, it will require formulating so as to ensure colonic delivery. Without employment of a colonic delivery platform our data might predict that chronic consumption of Manuacol LD could lead to iron deficiency, due to iron chelation within the small bowel. In the context of modulating the intestinal microbiome, it is known that iron is able to alter microbial colonisation to a more pro-inflammatory enterotype

and it could be envisaged that Manuacol LD could suppress the colonisation of these 'non-beneficial' bacteria (such as *Bacteroides*) through the chelation of luminal iron.[11, 43, 44] Further to this, alginates may have pre-biotic effects acting as a support scaffold for the colonisation of beneficial bacteria and as such further testing of these pro- and pre-biotic effects in man is required.

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## **AUTHOR CONTRIBUTIONS**

RH, GOL-D, MS, MS, AB and CL performed the experiments. RH, GOL-D, SEH, TI, IN and CT conceived and designed the experiments. RH, GOL-D and SEH analysed the data. RH and CT wrote the paper.

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## **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## TABLES

**TABLE 1** Summary of analytical ultracentrifugation data to determine alginate molecular weight (kDa) and nuclear magnetic resonance spectroscopy to determine alginate G:M composition.

<b>Alginate</b>	<b>MW (kDa)</b>	<b>G:M</b>
RF	230 ± 10	60:40
KEL	220 ± 15	46:54
GHB	180 ± 18	53:47
DH	170 ± 6	40:60
AFH	155 ± 5	29:71
LD	145 ± 5	38:62
LFR	74 ± 3	62:38

## FIGURE LEGENDS

**FIGURE 1** (A) Ferritin protein expression in RKO cells co-cultured with iron (Fe) +/- sodium alginates (LD, DH, GHB, LFR, KEL, RF and AFH) for 24 hours. Data points represent mean fold change in protein expression normalised to  $\beta$ -actin, relative to control. (B) Ferritin protein expression in RKO cells co-cultured with different iron concentrations (Fe) +/- LD, DH, GHB, LFR, KEL, RF and AFH for 24 hours. Data points represent mean fold change in protein expression normalised to  $\beta$ -actin, relative to control. (C) Total iron concentrations in RKO cells cultured +/- Manuacol LD for 24 hours. (D) Transferrin receptor expression in RKO cells incubated with iron +/- Manuacol LD. Data points represent mean fold change in protein expression, normalised to  $\beta$ -actin, relative to control. Error bars denote  $\pm$  SEM, \* represents statistical significance  $p < 0.05$  vs iron only control and  $n = 3$ .

**FIGURE 2** (A) Iron concentration in Caco-2 cells incubated with iron +/- Manuacol LD. (B)  $^{59}\text{Fe}$  concentrations at 0.5, 4 hours and 24 hours in the apical chamber following culture with iron +/- Manuacol LD. Error bars denote  $\pm$  SEM, \* represents statistical significance,  $p < 0.05$  vs iron only control.

**FIGURE 3** (A) Whole carcass (total absorbed iron across the gut)  $^{59}\text{Fe}$  CPM concentrations 48 hours post administration with iron +/- Manuacol LD. (B) Faecal  $^{59}\text{Fe}$  CPM 48 hours post administration with iron +/- Manuacol LD. (C) Stomach, duodenum and colon  $^{59}\text{Fe}$  CPM concentrations 48 hours post administration of iron +/- Manuacol LD. (D) Total accountable  $^{59}\text{Fe}$  CPM concentrations for each experimental group compared to the dose concentration of  $^{59}\text{Fe}$  administered. Error bars denote  $\pm$  SEM, \* and \*\* represents statistical significance  $p < 0.05$  and  $0.005$  vs iron only control.

**FIGURE 4** (A) Manuacol DH relative viscosity ( $\eta_{rel}$ ) decreases by heating at  $100\text{ }^{\circ}\text{C}$  with a plot of intrinsic viscosity against heating time. Error bars denote  $\pm$  SEM. (B) Ferritin protein expression in RKO cells incubated with iron +/- Manuacol DH

heat degradation products for 24 hours. Data points represent mean fold change in protein expression, normalised to  $\beta$ -actin, relative to control. Error bars denote  $\pm$  SEM, \* represents statistical significance  $p < 0.05$  vs iron only control and  $n = 3$ . **(C)**  $^{59}\text{Fe}$  concentrations in RKO cells incubated with iron challenged +/- DGA . Data points represent mean fold change in protein expression, normalised to  $\beta$ -actin, relative to control. Error bars denote  $\pm$  SEM. NS indicates no statistical significance. **(D)** Ferritin protein expression in RKO cells incubated with iron +/- DGA. Data points represent mean fold change in protein expression, normalised to  $\beta$ -actin, relative to control. Error bars denote  $\pm$  SEM. NS indicates no statistical significance.

**FIGURE 5** Experimental (red) and simulated (blue) NMR spectra of alginate **(A)** Manucol DH and **(B)** Manucol LD for the region of protons 1 and 5 of mannuronic acid and glucuronic acid. **(C)** CD spectra and representative UV-Visible spectra of Manucol LD and Epimerised Manucol LD. **(D)** Ferritin expression in RKO cells incubated with iron +/- Manucol LD and EpLD. Data points represent mean fold change in protein expression, normalised to  $\beta$ -actin, relative to control. Error bars denote  $\pm$  SEM, \* represents statistical significance  $p < 0.005$  vs iron only control and  $n = 3$ .