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Evidence for an Enhancing Effect of Alginate on Iron Availability in Caco-2 Cells

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ABSTRACT: The potential use of alginates as a vehicle for water-soluble (bioavailable) iron for fortifying food products was examined using a Caco-2 cell model system. Cell monolayers were exposed to alginates with various mannuronic to guluronic acid ratios at three different concentrations, and cellular ferritin was measured as a surrogate marker of iron uptake into the cell. Ferritin concentrations were significantly higher when the cells were treated with ferric ammonium citrate and 0.5 and 1% w/v (but not 0.1%) alginate, but were unaffected by mannuronic/guluronic acid ratios. The enhancing effect of ascorbic acid was maintained with 0.1% alginate and significantly increased with 0.5 and 1% alginate, whereas the inhibitory effect of tannic acid was significantly reduced with 0.5% alginate. Alginate beads delivered available iron to Caco-2 cells, indicating that they are a promising vehicle for soluble iron with potential use in food fortification programs.

KEYWORDS: iron bioavailability, alginate, Caco-2 cells, ferritin, ascorbic acid, tannic acid

INTRODUCTION

Estimates made by the World Health Organization suggest that anemia affects 1.62 billion people, that is, one-fourth of the world's population, with preschool children and pregnant women being at greatest risk.[1] Because iron deficiency is a common cause of anemia, fortification of foods with iron is considered to be a sustainable and realistic way to reduce the risk of iron-deficiency anemia[2] but is a challenge for the food industry. This is because water-soluble forms of iron, which are generally more bioavailable than nonsoluble iron compounds,[3] often cause adverse organoleptic changes when added to foods.[4] A potential strategy for overcoming this problem is the use of water-soluble iron compounds that are protected by a water-resistant barrier. Alginates, natural biopolymers present in brown seaweed, have been shown to bind divalent and trivalent cations[5,6] and can form a stable complex with ferric iron over a range of different pH values.[6] They may therefore be a useful vehicle when water-soluble iron compounds are employed to fortify food products.

A model system commonly used for an iron bioavailability screening employs Caco-2 cells.[7−9] Once differentiated, these cells share many characteristics of normal absorptive enterocytes.[10,11] Furthermore, if a food/compound is subjected to simulated gastric and small intestinal digestion, with the associated changes in pH,[12] before application to the Caco-2 model system,[8] the similarity to the in vivo situation and its predictive value improve.[13,14] This system is a valuable tool for screening different iron sources to rank them for potential bioavailability and for investigating mechanisms of absorption in humans.[7,15]

Alginates are composed of various ratios of two different acids, D-mannuronic and L-guluronic acid (M:G), and due to the variable length of the polymer chains, they exhibit wide physicochemical properties[16] and are used in a wide variety of applications, including drug delivery systems,[17] antireflux preparations,[18] and thickening agents in food products.[19] The effect of alginates, used as thickening agents in infant formulas, on iron, zinc, and calcium availability has been investigated in vitro; the bioavailability of iron and zinc increased, whereas that of calcium decreased in the presence of alginates.[20] In comparison, results from a study carried out in six ileostomy subjects showed that alginates, when administered in frozen milkshake and jam, had no significant effect on iron absorption, but the study was underpowered.[21]

The aim of the present study was to evaluate the effects of food grade sodium alginates (E401) on iron uptake into Caco-2 cells. We designed experiments to determine if they could be used as a delivery system (protective barrier) for soluble iron. Specifically, Caco-2 cell studies were carried out to evaluate the effects of various ratios of mannuronic and guluronic acids on iron uptake from ferric ammonium citrate (FAC) and ferrous gluconate (FeG) in the presence of an iron enhancer, ascorbic acid (AA), and an iron inhibitor, tannic acid (TA).

MATERIALS AND METHODS

Caco-2 Cell Culture Procedures. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, UK. Caco-2 cells (HTB-37) were obtained from American Type Culture Collection (Manassas, VA, USA) at passage 20 and stored in liquid nitrogen. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen UK; supplemented with 10% fetal bovine serum, 25 mM HEPES solution, 4 mM L-glutamine, and 5 mL 5000 u/mL penicillin/streptomycillin solution) and were maintained at 37 °C in an incubator with humidified atmosphere consisting of 5% carbon dioxide and 95% air.

Caco-2 cells were seeded onto collagen-coated 6-well plates (Greiner, UK) at a density of 4.75×10^4 and suspended in 2 mL of supplemented DMEM, which was replaced every 2 days. Cells between passages 32 and 38 were used for experiments at 13 days post seeding, and 24 h prior to experimentation, cells were switched to serum-free medium (DMEM supplemented as above with the exception of fetal bovine serum). Experiments without a simulated digestion phase were initiated by the addition of fresh serum-free medium containing the appropriate treatment. After 24 h, the treatment medium was aspirated from the 6-well plates, and the cells were washed twice with 2 mL of PBS and, subsequently, deionized water was applied to each well and the Caco-2 cells were scraped off using an inverted 200 μL pipet tip. The cell suspension from each well was transferred into 5 mL sample tubes and sonicated on ice, three times for 5 s, using a probe sonicator. Following sonication, cell suspensions were transferred to prelabeled 2 mL sample tubes and stored at −20 °C.

Experiments using iron-containing alginate beads began with a simulated digestion phase,[8,12] but all other conditions were similar apart from the ones listed below: serum-free DMEM (Gibco, UK) was substituted with minimum essential medium (MEM),[13] and the incubation time with the digests (to imitate the duodenal phase) was 2 h, after which time the digests were removed and the cells incubated for a further 22 h.

Sonicated cell lysates were defrosted at room temperature and kept on ice, and a spectroferritin ELISA assay (Ramco, USA) was carried out 24 h postsonication, according to the manufacturer's instructions. Total protein was quantified using a BCA protein assay (Pierce, USA). Uptake of FAC or FeG by Caco-2 cells was measured from the ferritin content (ng/mg total protein), a surrogate index of iron availability.

Preparation of Alginate Solution in Serum-free Medium. Three alginate formulations with different ratios of mannuronic to guluronic acid (M:G) were used in this study: A1 (Manucol DH, M:G ratio 60−70:30−40), A2 (Manugel GHB, M:G ratio 30−40:60−70) from FMC BioPolymer, Haugesund, Norway, and A3 (D1, M:G ratio 60:40) from Danisco, Copenhagen, Denmark. All glassware and magnetic stirrers were washed in 10% hydrochloric acid and rinsed with deionized water prior to use to eliminate iron contamination. The alginate solutions were prepared by dissolving 3 g of alginate powder in 300 mL of serum-free medium to obtain 1% (w/v) stock solutions. The solution was covered with sterilized parafilm and left overnight at room temperature with stirring. If needed, the stock solutions were diluted further in serum-free medium to obtain working solutions of 0.5 or 0.1% (w/v) alginate concentrations, which were applied to the Caco-2 cells in media containing ferric ammonium citrate (FAC) (30 μmol/L), with or without L-ascorbic acid (AA) (600 μmol/L) or tannic acid (TA) (molar ratio of 1:0.1 FAC:TA).

Preparation of Ascorbic Acid, Tannic Acid, Ferric Ammonium Citrate, and Ferric Chloride Solutions. Immediately before use, L-ascorbic acid solution was prepared at a concentration of 0.06 mol/L (1/100 dilution of concentrated stock) in Milli-Q water. A working solution of 0.0006 mol/L was prepared in the treatment medium. Ferric ammonium citrate (FAC) or ferric chloride ($FeCl_3$) solutions were prepared at a concentration of 0.003 mol/L (1/100 dilution of concentrated stock) in 0.1 or 1 mol/L HCl, respectively. Working solutions were prepared in relevant treatment media. Tannic acid solutions were prepared at a concentration of 0.001 or 0.0003 mol/L (1/100 dilution of concentrated stock). Working solutions at a final Fe:TA ratio of 1:0.1 were prepared in treatment medium. All solutions were filter sterilized using hydrophilic 0.2 μm syringe filters (Sartorius Stedim Biotech, Germany).

Preparation of Treatment Media. Immediately before use, 1% (w/v) alginate solution and serum-free media were heated to 37 °C. Subsequently, the treatment medium solutions were prepared in 50 mL tubes under sterile conditions by the addition of appropriate volumes of 1% alginate solution (to obtain required concentrations of 0.1, 0.5, or 1% (w/v) in serum-free media solutions) followed by the addition of iron solutions (FAC or $FeCl_3$), TA, or AA solutions to obtain the final concentrations of iron (30 μmol/L), TA (at 1:0.1 molar ratio of iron to TA), or AA (600 μmol/L). Shortly before the

start of an experiment, serum-free medium was aspirated from the cell monolayer, 2 mL of the required treatment medium was applied to each well and incubated (37 °C, 5% CO_2) for 24 h, and then the cells were harvested.

Preparation of Alginate Beads. Alginate beads were prepared using an encapsulator (EncapBioSystemS Inc., Switzerland) from 0.5% (w/v) alginate solution. The alginate beads were placed in a calcium and iron (FAC or FeG) bath where cross-linking took place, resulting in iron-loaded alginate beads. The iron content of the beads was measured by atomic absorption spectroscopy.[22]

Simulated Digestion Phase. Alginate beads and the positive control ($FeCl_3$ with AA, as detailed in Table 1) underwent a simulated

Table 1. Composition of Alginate Beads

beads	iron compd added[a]	wt of beads, g	iron/ascorbic acid ratio	iron content, μg	calcium content, μg
	$FeCl_3$ + AA		1:2	8.38	
B1	FAC	0.049		8.38	data not available
B2	FeG	0.044		8.38	data not available
B3	FeG	0.174		8.38	data not available
B4	FeG	0.419		8.38	data not available
	$FeCl_3$ + AA		1:10	8.38	
B5	FeG	0.044		8.38	16.7
B6	FeG	0.093		8.38	65.1
B7	FeG	0.175		8.38	85.8
B8	FeG	0.419		8.38	289.1
B9	FeG	0.209	1:10	8.38	108.7
B10	FeG	0.419	1:10	8.38	217.9

[a]FAC, ferric ammonium citrate; FeG, ferrous gluconate.

digestion[8,12] phase prior to application onto the dialysis membrane placed above the Caco-2 cell monolayer. Briefly, samples under investigation were first exposed to pepsin digestion for 1 h in an acidic environment (pH 2) at 37 °C on a rolling table to represent gastric conditions. Once the first phase was completed, the pH was gradually adjusted to pH 6.7,[14] and pancreatic bile solution was added to the samples followed by further pH adjustment to 6.9−7; the digest was applied onto dialysis membranes placed above the Caco-2 monolayer and incubated for 2 h at 37 °C. The digestate was then removed from the upper compartment, and the Caco-2 cells were left for a further 22 h to allow ferritin expression to take place.[12]

Statistical Analysis. Unless otherwise stated, all statistical analyses were performed using SPSS Inc., USA (version 16.0.0). Two-factor ANOVA with Tukey posthoc or Dunnett (two-sided) t test were conducted to examine pairwise differences on log-transformed data. Data are presented as the mean ± SD. Differences were considered to be significant at $p < 0.05$.

Each table or figure represents data from one experiment. The number of replicates performed in each experiment is indicated as n and is provided in figure captions or table footnotes.

■ RESULTS AND DISCUSSION

Effect of Alginates on Ferritin Concentration. There was no effect of 0.1% (w/v) A1, A2 (Figure 1), or A3 (Figure 2) alginate on FAC-induced ferritin formation in Caco-2 cells, but higher concentrations (0.5 and 1%) resulted in a significantly higher ferritin expression ($p < 0.05$). In cells exposed to FAC (30 μmol/L) there was a >10-fold increase in cell ferritin concentrations when compared to untreated control cells (ferritin concentration = 4.7 ng/mg protein). These data demonstrate that alginates can increase iron bioavailability in this in vitro system. There was a similar trend in response
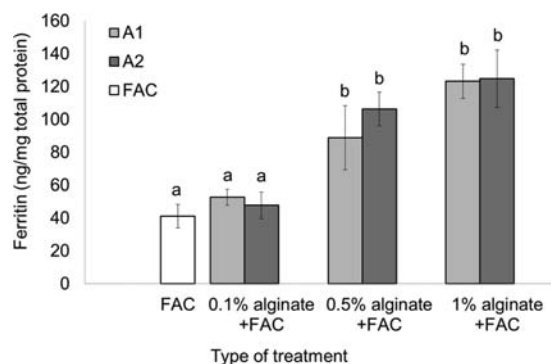
Figure 1. Iron uptake in Caco-2 cells exposed to ferric ammonium citrate (FAC) and different alginates. Ferritin concentration (ng/mg total protein) after treatment with 30 μmol/L FAC, FAC + A1, or FAC + A2 at 0.1, 0.5, and 1% (w/v) concentrations. Data represent the mean ± SD (n = 4; except for FAC, where n = 6). Bars without a common letter (a, b) are significantly different, $p < 0.05$.
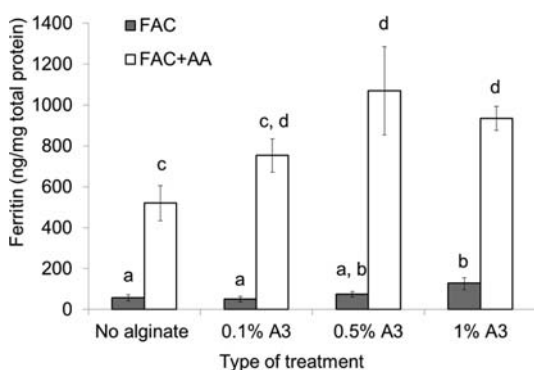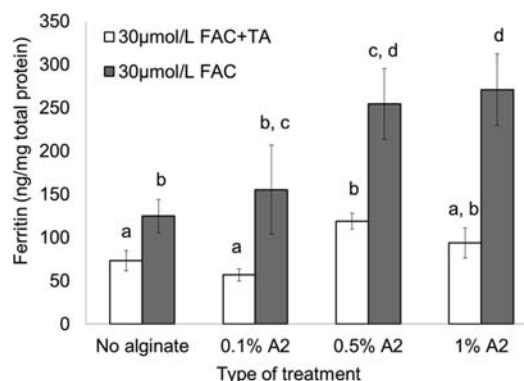

Figure 2. Iron uptake in Caco-2 cells exposed to ferric ammonium citrate (FAC) and different alginate concentrations and ascorbic acid. Ferritin concentration (ng/mg total protein) after treatment with 30 μmol/L FAC (n = 3), FAC plus A3 at three concentrations (0.1, 0.5, and 1% w/v) (n = 4 for each alginate concentration used, black bars), FAC and ascorbic acid (AA at 600 μmol/L) (n = 3), or FAC and A3 and AA (n = 4 for each alginate concentration used, white bars). Data represent the mean ± SD. Bars without a common letter (a–d) are significantly different, $p < 0.05$.

(Figures 1 and 2) when the cells were treated with three different types of alginate (A1, A2, and A3), indicating that the M:G ratios in the alginates tested did not have an effect on ferritin formation.

Effect of Ascorbic Acid (AA) and Alginate on Ferritin Concentration. As predicted, cells exposed to FAC plus AA had a significantly higher ferritin concentration than cells incubated with FAC alone ($p < 0.01$, Figure 2). Interestingly, ferritin formation was increased further in the presence of 0.5 and 1% A3 alginate when compared to AA alone ($p = 0.01$ and $p < 0.05$, respectively).

Effect of Tannic Acid (TA). It has previously been reported that at 1:0.1 (Fe:TA molar ratio) TA inhibits iron uptake into Caco-2 cells by 92%.[23] Co-addition of TA significantly inhibited ferritin formation induced by FAC at 30 μmol/L ($p < 0.05$; Figure 3). When 0.5% A2 alginate was added with iron and TA, the ferritin formation was restored to levels obtained in samples treated with iron alone, resulting in significantly higher ferritin concentrations than in cells treated with iron and TA alone ($p < 0.01$). However, the inhibitory effect of TA was not blunted at low or high (0.1 or 1% w/v) alginate concentrations (Figure

Figure 3. Iron uptake in Caco-2 cells exposed to ferric ammonium citrate (FAC), alginate, and tannic acid. Ferritin concentration (ng/mg total protein) after treatment with 30 μmol/L FAC or FAC and A2 at 0.1, 0.5, and 1% (w/v) concentrations, with or without tannic acid (TA) at iron/TA molar ratio 1:0.1. Data represent the mean ± SD (n = 4). Bars without a common letter (a–d) are significantly different, $p < 0.05$.

3). Similar results were observed at higher iron concentrations (100 μmol/L FAC) and TA (data not shown). These results suggest that an alginate concentration of 0.5% (w/v) was the most favorable in terms of negating the inhibitory effect of TA.

Effect of Iron-Containing Alginate Beads. Experiments were performed to examine the stability of alginate beads containing various levels of alginate (Table 1) but a constant amount of ferrous gluconate (FeG) when subjected to simulated digestion. The results showed that FeG was more effective in inducing a ferritin response in Caco-2 cells than ferric ammonium citrate (Table 2).

Table 2. Ferritin Concentration in Caco-2 Cells Treated with Alginate Bead Formulations (B1−B4) Containing Different Forms of Iron in the Presence of AA

type of treatment	ferritin ± SD,[a] ng/mg total protein
Fe + AA	13.6 ± 3.3 ab
B1	8.4 ± 1.2 a
B2	13.5 ± 3.0 ab
B3	15.0 ± 2.8 b
B4	15.4 ± 2.9 b

[a]Data refer to cells treated with ferric chloride and ascorbic acid (Fe + AA), ferric ammonium citrate (FAC) loaded beads (B1), or ferrous gluconate loaded beads (B2, B3, B4). Data represent the mean ± SD (n = 4). Means without a common letters (a, b) are significantly different, $p < 0.05$.

Ferritin formation in cells treated with digests of B6 beads (containing 0.093 g of alginate) was at the level of the positive control (FeCl$_3$:AA) (Table 3). Increasing the alginate content of the beads (B7, B8) inevitably increased the calcium content, and this resulted in a significantly lower ferritin concentration. However, addition of AA during bead formation (i.e., B9, B10) counteracted the inhibitory effects observed in B7 and B8 ($p < 0.01$, Table 3). Furthermore, AA containing beads B10 produced a significantly greater ferritin response than AA alone ($p = 0.01$), suggesting a synergistic effect between AA and alginate, which warrants further investigation.

The mechanism by which alginates enhance iron uptake into Caco-2 cells remains uncertain. On the basis of the fact that alginates bind calcium cations,[5,24] we hypothesize that when

Table 3. Ferritin Concentration in Caco-2 Cells Treated with Alginate Beads Containing Ferric Chloride (B5−B10)

type of treatment	ferritin concentration ± SD,[a] ng/mg total protein
Fe:AA 1:10 ratio	73.8 ± 13.1d
B5	34.3 ± 9.9 bc
B6	57.5 ± 16.8 cd
B7	16.9 ± 5.5 a
B8	25.1 ± 8.6 ab
B9 + AA Fe:AA 1:10 ratio	84.6 ± 30.1 d
B10 + AA Fe:AA 1:10 ratio	155.5 ± 17.6 e

[a]Data refer to cells treated with ferric chloride and ascorbic acid (Fe/AA) at 1:10 ratio, iron loaded alginate beads (B5−B8), or iron-loaded alginate beads and AA at 1:10 ratio (B9, B10) Data represent the mean ± SD ($n = 4$). Means without a common letter (a−d) are significantly different, $p < 0.05$.

alginates are added to the culture media they form a complex with calcium, a recognized inhibitor of iron absorption,[25] which is present in the media as calcium chloride (at a concentration of 200 mg/L). High concentrations of calcium can act as a noncompetitive inhibitor of iron absorption via DMT1[26] and, in addition, can induce translocation of DMT1 away from the cell surface of Caco-2 cells.[27] A limitation of our model system is that calcium (an essential component of cell culture media) cannot be removed without deleterious effects on the cell monolayer. Furthermore, attempts at increasing the calcium content in culture media in the presence of alginate failed due to the immediate formation of a thick gel.

In conclusion, our results demonstrate that alginates increased the uptake of iron into Caco-2 cells and enhanced the effect of AA, demonstrating that they have a positive effect on iron bioavailability. The efficiency of iron uptake into Caco-2 cells depends on the calcium content and chemical form of iron in the alginate beads. Alginates also protected iron from the inhibitory effects of TA under the conditions tested. Alginate beads could therefore be used in food fortification programs as a delivery vehicle for iron. Future work will focus on bead stability in food products with various water activities.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AA, ascorbic acid; DMEM, Dulbecco's modified Eagle's medium; FAC, ferric ammonium citrate; FeG, ferrous gluconate; HCl, hydrochloric acid; IDA, iron deficiency anemia; MEM, minimum essential medium; M:G, D-mannuronic to L-guluronic acid ratio; TA, tannic acid.

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