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Heme Iron Release from Alginate Beads at *In Vitro* Simulated Gastrointestinal Conditions

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Abstract Heme iron (Fe) release from alginate beads at *in vitro* simulated gastrointestinal conditions for potential use as oral heme Fe supplement was studied. Five beads at different ratios of sodium alginate (SA)-to-spray-dried bovine blood cells (SDBC) with weight ratios of 1:1.25, 1:2.5, 1:5, 1:10, and 1:15 (*w*/*w*) were prepared. Release characteristics of these beads were investigated at *in vitro* simulated gastrointestinal conditions. Release media pH strongly influenced the controlled Fe release from the beads. The heme Fe-beads in simulated gastric fluid (pH 2) remained in a shrinkage state and Fe release was low: 25.8, 21.1, 11.6, 12.1, and 12.0 % for 1:1.25, 1:2.5, 1:5, 1:10, and 1:15 ratios, respectively. Proportion and amount of Fe released by 1:1.25 and 1:2.5 ratios was higher than the other ratios. The heme Fe-beads swelled and dissociated in simulated intestinal fluid (pH 6), releasing three-fourths of the Fe in 200 min. The morphology studies showed that Fe release followed formation of pores in the alginate matrix, generating erosion of the beads and complete disintegration after 75 and 200 min of gastric and intestinal incubation, respectively. These results indicate that heme Fe-beads may be useful for oral delivery of heme Fe supplement.

Keywords Heme iron · Alginate beads · Release · Gastrointestinal conditions

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Introduction

Fe deficiency anemia is the most common nutritional deficiency in the world today. The World Health Organization (WHO) has reported that Fe deficiency manifests as anemia in up to 2 billion people, affecting about 30 % of population [30]. One of its main causes is poor dietary intake of bioavailable Fe (heme Fe), which constitutes 95 % of functional Fe in the human body [11]. Heme Fe is an organic form (Fe^{2+}) of Fe present in the porphyrin molecule of hemoglobin or myoglobin, and derived almost exclusively from animal food sources (beef, lamb, pork, meat products, and viscera) [15, 27]. Heme Fe has a greater bioavailability that non-heme Fe (inorganic Fe) [3]. Body Fe is maintained primarily by regulation of the absorption of dietary Fe in the proximal small intestine. Heme Fe and non-heme Fe enter the enterocyte by independent pathways [4]. Heme Fe is more efficiently absorbed (15–35 %) than non-heme Fe (2–20 %) [17]. However, meat products as heme Fe sources are expensive and not available for a sizable part of people in developing countries.

Encapsulation technology has been used to protect non-heme Fe, reducing its precipitation and chelation reactions with other diet components in the gastrointestinal tract, with promising results [13, 33]. We have used spray-dried bovine blood cells (SDBCs), a by-product obtained from slaughter plants by erythrocyte fractionation and spray drying. This product is high in protein content (90 %), hemoglobin concentration heme Fe. Recently, our research group reported that this product increases heme Fe absorption from the intestine; we have named it 'erythrocyte stroma factor' [22]. This product was used to develop heme Fe-alginate beads reported in previously published work [28]. Alginates are natural polysaccharides obtained from brown algae; they consist of linear chain residues of β-D-mannuronic acid and α-L-guluronic acid in different proportions. Alginates are used in a wide range

of applications, particularly for the encapsulation of food and pharmaceutical substances. They have capacity to form gels, and can generate beads by ionic gelation when a solution of sodium alginate (SA) is dropped into a divalent cation solution [6].

Currently, the information on heme Fe encapsulation to generate an oral Fe supplement is limited. The studies that exist have used the methodology of entrapment in liposomes [31, 32]. But according to our knowledge, there is no research about other methods of encapsulation for this type of Fe for the prevention or treatment of Fe deficiency anemia. In this study, we report an assessment of using alginate beads to deliver heme Fe in an *in vitro* gastrointestinal conditions.

Material and Methods

Material

SDBC from bovine were purchased from Licán Alimentos S.A., Santiago, Chile; and were used as core material. SA (viscosity of 25.7 cps at 25 °C, 2 g/100 mL solution) was purchased from Sigma-Aldrich, USA, and was used as wall material. Calcium chloride, pepsin, and all other reagents of analytical grade were purchased from Merck S.A. Bile extract and pancreatin (trypsin, amylase, lipase, ribonuclease, and protease) were purchased from Sigma-Aldrich, USA.

Fe Content and Surface Charge of SDBC

Total Fe content of SDBC was determined using an atomic absorption spectrophotometer GBC, 905AA, Australia, after acid digestion (method 999.11) [2]. Non-heme Fe was obtained by acid extraction [24] and quantified with atomic absorption spectrophotometry. The spectrophotometric measurements were according to a standard curve assessed at λ = 248.3 nm, using a commercial Fe standard, 1000 μg/mL (J.T. Baker, USA). Heme Fe content was calculated from the difference of total Fe and non-heme Fe. These procedures were done in triplicate.

Heme Fe-Beads Preparation

The heme Fe-beads were prepared according to Valenzuela et al. [28] as follow: SDBC were suspended in SA solution (2 % *w*/*v* in deionized water) at wall-to-core weight ratios of 1:1.25, 1:2.5, 1:5, 1:10, and 1:15 (*w*/*w*). These suspensions were dropped from a tuberculin syringe into gelling solution of calcium chloride (5 % *w*/*v* in deionized water). The beads were formed instantly and were deposited in plastic boxes and dried to a constant weight at 40 °C (≈10 h). The dried beads were removed from the boxes and stored at environmental conditions. Total Fe, heme Fe, and calcium (Ca) content of the beads were determined by atomic absorption spectrophotometry [2]. The spectrophotometric measurements for Ca were according to a standard curve assessed at λ = 422.7 nm.

In Vitro Release Studies

To simulate the human digestive tract, two conditions were used: gastric fluid (GF) and intestinal fluid (IF). The GF consisted of 2 g/L of NaCl containing 10 g/L of pepsin with pH adjusted to 2.0 with HCl 1 N. Then, 2.5 g of beads were mixed in 100 mL of GF and incubated for 75 min at 37 °C with constant agitation at 150 oscillations/min. IF was prepared by dissolving 50 g/L of pancreatin and 31.2 g/L of bile extract in intestinal solution (8.76 g/L NaCl and phosphate buffer saline (PBS) 0.1 M at pH 7.4). The pH was adjusted to 6.0 with HCl 1 N. Beads from GF incubation were collected by filtration with a strainer and dried on absorbent paper. These beads were weighed and incubated in 100 mL of IF for 200 min at 37 °C with constant agitation at 150 oscillations/min. The release pattern of total Fe was measured at each step (GF and IF medium) directly from aliquots of 5 mL taken each 15 and 20 min for gastric and intestinal incubation, respectively. The total Fe content was measured by atomic absorption spectroscopy. These procedures were done in triplicate.

The cumulative Fe release, at GF and IF condition, from beads with wall-to-core weight ratios of 1:1.25, 1:2.5, 1:5, 1:10, and 1:15 (*w*/*w*) were best fitted mathematically with a quadratic function: $y = a + b_0x\text{-}b_1x^2$, where $a = 0$, b_0 = % Fe released/unit of time, and b_1: % Fe released/(unit of time)^2.

Disintegration of Heme Fe-Beads

Disintegration of the beads was performed according to Anal and Stevens [1] with some modifications. Beads (≈1 g) were preincubated with 50 mL of GF; after filtering, the beads were incubated in 50 mL of IF, until complete disintegration of the beads was achieved. The time of disintegration was registered in minutes. All disintegration experiments were done in triplicate.

Morphology by Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy and Transmission Electron Microscopy

At the end of the incubation in GF and after 120 min of incubation in IF, 0.5 g of beads were drained and dried on adsorbent paper and processed for observation by scanning electron microscopy (SEM) on a LEO 1420 VP, UK equipped with an energy dispersive X-ray spectroscopy (EDS) at 25 kV. Prior to observation, the beads were dehydrated through an acetone series and dried by means of a critical point dryer, and then mounted on a cylindrical aluminum stub, upon which the

beads were fixed using double-sided tape. The beads were then gold-sputter-coated twice at 20 kV in an argon atmosphere (PELCO 91000) to render them electrically conductive.

Bead fragments obtained after disintegration were drained and fixed in 2.5 % glutaraldehyde in sodium cacodylate buffer 0.1 M at pH 7.0. They were dehydrated in ethanol 50, 70, 95, and 100 % for 15 min each and embedded in epoxy resin:ethanol (1:1) overnight and then were included in the epoxy resin alone. The resin was polymerized at 60 °C for 24 h. Thin sections were obtained from Sorvall MT-5000 ultramicrotome and stained with aqueous uranyl acetate 1 % for 1 min and observed with transmission electron microscopy (TEM) (Philips Tecnai 12 BioTwin, Netherlands) operated at 80 kV. The photographs were taken with Megaview G2 Snapshot software.

Swelling Studies

One gram of the pre-weighed dry beads was placed in a plastic petri dish and immersed in 20 mL of GF at pH 2 and PBS adjusted to pH 6 with HCl 1 N at 37 °C. Weight change of the beads was monitored at 1 and 2 h for GF and PBS buffer, respectively, as follows: the beads were separated from the medium using a stainless steel grid. Immediately, they were wiped gently with paper and weighed. The percentage of weight change of the beads was calculated from the following equation (Eq. 1). These procedures were done in triplicate.

$$\text{Weight change}\% = \left(\frac{\text{FW} - \text{IW}}{\text{IW}}\right) \times 100 \quad (1)$$

Where FW is final weight, IW is initial weight.

Surface Area Determination

The diameter of the dry beads was determined with a digital micrometer (Veto E5010109, China) ($N = 50$ by each replicate). The surface area of the beads was calculated from the following formula (Eq. 2).

$$\text{mm}^2 = \Pi \times r^2 \quad (2)$$

Statistical Analysis

The results were processed by ANOVA and Tukey post hoc test. The analysis of surface area was processed by Kruskal-Wallis and all pairwise comparisons test. Statistix 8 was used for statistical analyses. Differences between means were considered significant at $p < 0.05$.

The results from *in vitro* release studies at GF and IF conditions were characterized by mathematical functions, and the b_0 coefficients from quadratic function from each treatment were compared with t test ($p < 0.05$) using SPSS 15.0 program.

Results and Discussion

Fe Load and Release from Heme Fe-Beads at Gastric Conditions

SDBC has a high content of heme Fe (2412 ± 196 μg/g), which represents 99 % of the amount of total Fe. For this reason, Fe release measurements were quantified as total Fe, because it is a simple and rapid method. As shown in Table 1, the heme Fe content of the beads increased significantly with increasing SDBC concentration, as expected [28]. It was not possible to compare the heme Fe content of the beads prepared in this study with the heme liposomes developed by Yuan et al. [31] because these authors did not report this value. Perez-Moral et al. [21] elaborated non-heme Fe-alginate beads that showed a higher Fe content of (50–80 mg Fe/g dried bead) than the heme Fe-beads of this work, because the major Fe concentration of the non-heme Fe sources differed.

The Ca content of the beads was similar for all the bead ratios (Table 1). The ratio Ca:heme Fe is shown in Table 1, where it can be seen that the Ca:heme Fe ratios were decreased as heme Fe content increased, as expected. While it is true that Ca has been described as an inhibitory factor of heme Fe absorption in human [9, 10], Hallberg et al. [9] established that the doses of Ca required to generate this effect are from 300 to 600 mg. However, Hallberg et al. [10] in a later study indicated that 165 mg Ca as $CaCl_2$ added to a meal inhibited heme Fe absorption. In this case, the dose to cover human daily Fe requirements with the use of heme Fe-beads (1:15 ratio) is around of 12 g of beads which contain 40 mg of Ca. Hence, this low amount of Ca would not be expected to exert an inhibitory effect on the absorption of heme Fe because it is known that the effect of Ca on the heme Fe bioavailability is dose dependent, with a lower threshold of 40 mg of Ca [9]. It is important to clarify that the evidence for a Ca effect on human heme Fe absorption mainly comes from studies that did not isolate the effect of Ca from that of other dietary components because it was detected in single-meal studies [9, 10]. But currently, our research group described that a Ca dose of 800 mg ingested as $CaCl_2$ on an empty stomach diminished absorption of 5 mg heme Fe by 37.7 %. However, lower Ca doses did not affect the absorption of 5 mg heme Fe [5].

Several authors have studied the diffusion of various molecules encapsulated in alginate beads (drugs, proteins, bioactive compounds, minerals, etc.) in water, saline, or a variety of buffers [18, 23], but few have used physiological media with the addition of digestive enzymes. Figure 1a shows the cumulative release (percent) of Fe at gastric incubation, which was

Table 1 Properties of heme Fe-beads

Properties	Heme Fe-bead ratios				
	1:1.25	1:2.5	1:5	1:10	1:15
Heme Fe (μg/g beads)	71 ± 8[a]	147 ± 15[b]	369 ± 78[c]	605 ± 137[d]	811 ± 118[e]
Ca (μg/g beads)	2496 ± 164[a]	2935 ± 284[a]	3039 ± 333[a]	3253 ± 230[a]	3345 ± 43[a]
Ca:heme Fe ratio	35.2	20.0	8.2	5.4	4.1
Disintegration time (min)	150–200	142–190	132–210	147–220	138–200
Swelling in GF pH 2 (%)	−40 ± 4[a]	−35 ± 3[b]	−24 ± 3[c]	−13 ± 3[d]	−9 ± 4[d]
Swelling in PBS pH 6 (%)	53 ± 4[a]	35 ± 3[b]	32 ± 3[b]	29 ± 3[b,c]	25 ± 4[c]
Surface area (mm^2)	0.5 ± 0.1[a]	0.7 ± 0.2[a]	1.0 ± 0.3[b]	1.4 ± 0.4[c]	2.0 ± 0.7[c]

GF gastric fluid, *PBS* phosphate buffer saline. Means with different superscript letters are different ($p < 0.05$)

similar at the beginning of the study for all the beads. From 30 min of incubation (lag time), the Fe release from all types of beads increased gradually and was significantly lower at 75 min for 1:5, 1:10, and 1:15 ratios compared with 1:1.25 and 1:2.5 ratios. The low release of Fe from alginate beads at gastric condition has been reported by others [26]. The beads with higher concentrations of SDBC (1:5 to 1:15 ratios) lost only half of the Fe as the 1:1.25 and 1:2.5 ratios.

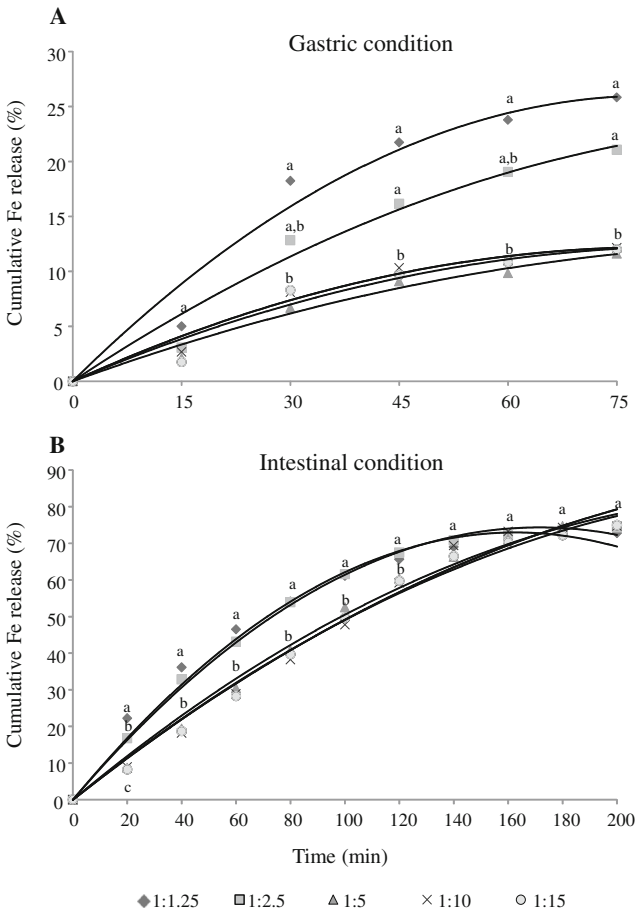

Fig. 1 Cumulative Fe release of heme Fe-beads at gastric and intestinal conditions. Means with different superscript letters are different ($p < 0.05$)

The quadratic function (Table 2) that characterizes Fe release from the beads reflects what is described above; the b_0 values from 1:1.25 and 1:2.5 ratios were different from b_0 of 1:15 ratios ($p < 0.05$).

The behavior of Fe release from the heme Fe-beads observed in this study may be explained by several factors mentioned as follows:

(1) Pore size: although the bead micrograph at 1:1.25 ratio showed a smooth surface in the native state before gastric incubation (Fig. 2a), in contrast to irregular surface of the 1:15 ratio (Fig. 2b), the 1:1.25 ratio presented larger pores in its surface (Fig. 1c) compared to 1:15 ratio (Fig. 1d) after gastric digestion. A greater magnification was needed to detect the pores on the surface of 1:15 ratio (Fig. 1d). Thus, the high Fe retention observed in the beads with the three highest proportions of heme Fe may be due to the smaller alginate pore size of these beads after gastric digestion, thereby slowing the rate of Fe release [8]. These finding are in accordance with Gombotz and Wee [7], who reported that the alginate and calcium ions react immediately in medium at acid pH, and a sponge-like matrix is formed from the outside to the

Table 2 Quadratic functions and r^2 of cumulative Fe release of heme Fe-beads at gastric and intestinal *in vitro* conditions

Heme Fe-beads	Gastric conditions	Intestinal conditions
1:1.25	$y = 0.6531^{a}\,x - 0.0041x^2$	$y = 0.8962^{ab}\,x - 0.0028x^2$
	$r^2 = 0.9628$	$r^2 = 0.9837$
1:2.5	$y = 0.4398^{ab}\,x - 0.0021x^2$	$y = 0.8669^{a}\,x - 0.0025x^2$
	$r^2 = 0.9678$	$r^2 = 0.9978$
1:5	$y = 0.2398^{bc}\,x - 0.0011x^2$	$y = 0.62^{c}\,x - 0.0012x^2$
	$r^2 = 0.9775$	$r^2 = 0.9924$
1:10	$y = 0.3017^{bc}\,x - 0.0019x^2$	$y = 0.5861^{bc}\,x - 0.0009x^2$
	$r^2 = 0.975$	$r^2 = 0.9853$
1:15	$y = 0.2809^{c}\,x - 0.0016x^2$	$y = 0.5935^{bc}\,x - 0.001x^2$
	$r^2 = 0.9499$	$r^2 = 0.9902$

Different superscript letters are different ($p < 0.05$)

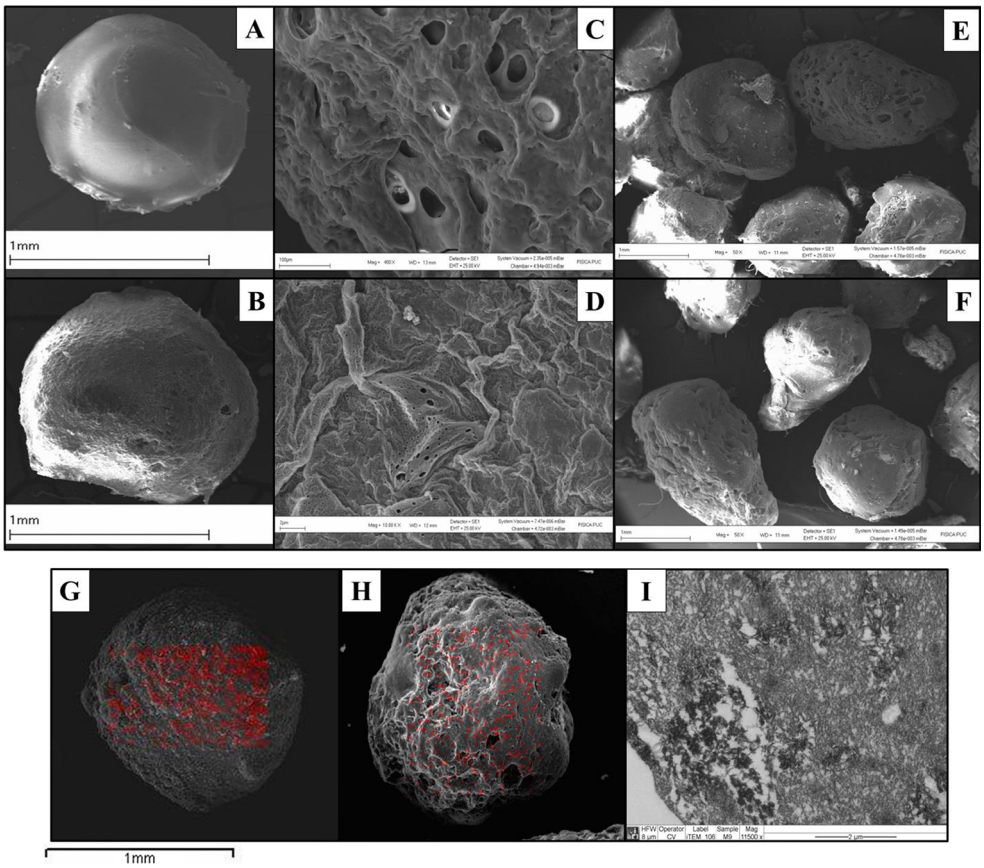

Fig. 2 SEM images of native beads 1:1.25 (**a**) and 1:15 (**b**) ratios; the same beads after gastric digestion (**c**, **d**), and 120 min post-intestinal digestion (**e**, **f**), respectively. SEM with EDS mapping of the native bead 1:15 ratio (**g**) (*red color* corresponds to Fe atoms), and the same bead after 120 min of incubation in intestinal fluid (**h**). TEM image of 1:15 bead fragments after incubation in intestinal fluid (200 min) (**i**)

inside of the beads, releasing the contents of these progressively.

(2) SDBC concentration: the greater amount of heme Fe can be extrapolated to a higher concentration of hemoglobin, which increases the viscosity of the bead core due to its gelling properties [28], and decreased Fe diffusion and release during gastric incubation in the beads with greater SDBC content [12].

(3) Surface area: the beads with higher Fe release in gastric conditions (1:1.25 and 1:2.5 ratios) showed a surface area significantly lower than the other beads (Table 1), thus may be more vulnerable to acid and/or pepsin attack, and degradation of the bead matrix. On the other hand, as surface area increases, the number of the apparent crosslinking points per alginate molecule could increase, retarding Fe release from alginate beads as demonstrated previously by Kim and Lee [14] for blue dextran alginate beads at larger sizes.

And finally, (4) pH: several authors have described the tendency of alginate beads to shrink when exposed to acidic environment (pH < 4) [19]. Shrinkage was observed in this work when the beads were incubated in GF (pH 2), showing negative swelling values (Table 1). Shrinking can be attributed to the electrical characteristics of the alginate molecules, which have a pKa around 3.5 and therefore tend to lose their negative charge at lower pH values [16]. The beads tended to shrink significantly less at higher SDBC concentration (Table 1); this higher shrinkage increased Fe release from the beads. These results could be explained by the observation of Pasparakis and Bouropoulos [20] who showed that core material release from alginate beads at acidic pH includes the expulsion of water (alginate beads typically contain up to 95 % water), and the diffusion of core molecules out of the beads as the acidic medium dehydrates the bead.

Fe Release from Heme Fe-Beads at Intestinal Conditions

The percent release of Fe in intestinal incubation is depicted in Fig. 1b. Before 120 min of intestinal incubation, the 1:1.25 and 1:2.5 ratios presented a higher Fe release, showing a behavior similar to that observed in the gastric incubation. The Fe release profile changed at 120 min of incubation, in which all the beads showed similar Fe release because these began to disintegrate showing a porous surface, elongated shapes, and detached fragments (Fig. 2e, f), generating a sustained and fast Fe release [25]. Then, the Fe release behavior is reflected in the quadratic function (Fig. 1b and Table 2); b_0 values from 1:1.25 and 1:2.5 ratios were different from b_0 of 1:5, 1:10, and 1:15 ratios ($p < 0.05$), showing that 1:1.25 and 1:2.5 ratios had a higher speed and total Fe release than the other treatments.

In Fig. 2g is shown the EDS mapping of a bead (1:15 ratio) before gastrointestinal incubation, which contains a greater Fe concentration (denoted with red color) than the same bead after its passage through the gastric and intestinal incubations photographed at 120 min (Fig. 2h). In Fig. 2h is shown the shape changes of the bead, an increase of protuberances, and surface deformation with pores of different sizes by gastrointestinal incubation effects.

Disintegration is shown in Table 1; the beads show an overall fragmentation at approximately 200 min. Disintegration occurs as the electrostatic interactions that maintain the beads in their native state became weakened or disappear at pH > 5 [7]. The beads swelled significantly (Table 1) and became dissociated rapidly, resulting in rapid Fe release. The increased swelling capacity under intestinal conditions (Table 1) has been reported by other authors, and can be explained by the alginate carboxyl groups tending to deprotonate at pH 6, decreasing electrostatic interactions that maintain the alginate network, allowing the medium to diffuse into the beads [23]. Moreover, at neutral pH values, there are increases in pore size, as shown in Fig. 2e, f, h, which facilitate the diffusion of molecules into and out of the beads [16]. However, all beads began to swell to varying degrees in relation to the SDBC amount, presumably caused by higher SDBC concentrations decreasing the electrostatic repulsive forces. This could cause the lower Fe release values from 1:5, 1:10, and 1:15 ratios (Table 1) before the disintegration phase of the beads began.

The fragments obtained from disintegrated beads were observed using TEM and are displayed in Fig. 2i. This technique was used to understand why 100 % of Fe is not released when the beads were completely disintegrated after gastrointestinal incubations. As shown in Fig. 2i, within the alginate network (light gray structure), it is possible to see the dispersion and location of SDBC inside the alginate network (dark gray structure), indicating that there is a low amount of SDBC remaining in the polymer network that may be released while the digestion process progresses.

The heme Fe-beads may be an appropriate delivery system for an oral heme Fe supplement that would have several advantages compared to other methods for treating iron deficiency anemia: (1) heme Fe from SDBC is absorbed by the enterocytes of the small intestine with higher bioavailability than sources of non-heme Fe [29], heme Fe as hemoglobin, heme Fe alone, or heme Fe plus animal proteins [22]. (2) The alginate beads help to bypass the acidity of gastric fluid without releasing substantial amounts of heme Fe, thus delivering high amounts of heme Fe to the small intestine. (3) Encapsulation allows combined sources of heme/non-heme iron to generate a more efficient dual supplementation. And (4) encapsulation reduces certain adverse organoleptic characteristics that Fe forms present, such as metallic flavor for non-heme Fe or 'taste of blood' for heme Fe.

Conclusions

The use of alginate for the formation of heme Fe-alginate beads resulted in favorable release of Fe at the intestinal level, where the Fe is absorbed. The 1:5, 1:10, and 1:15 heme Fe-alginate ratios released a low amount (≤12 %) of Fe at *in vitro* gastric conditions, and a high percentage (around 75 %) of Fe release under small intestine *in vitro* conditions. Heme Fe encapsulated in alginate beads is released by diffusion of Fe through the pores and by degradation of the alginate network. These results are favorable for future use of heme Fe-alginate beads as an oral Fe supplement; beads with greater amount of heme Fe (as 1:15 ratio) would be optimal for this purpose.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no competing interests.

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