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Maciel, VBV, Yoshida, CMP, Boesch, C et al. (2 more authors) (2020) Iron-rich chitosan-pectin colloidal microparticles laden with ora-pro-nobis (Pereskia aculeata Miller) extract. Food Hydrocolloids, 98. 105313. ISSN 0268-005X

https://doi.org/10.1016/j.foodhyd.2019.105313

IRON-RICH CHITOSAN-PECTIN COLLOIDAL MICROPARTICLES LADEN WITH ORA-PRO-NOBIS (Pereskia aculeata Miller) EXTRACT

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ABSTRACT

Iron deficiency represents a global nutrition gap that calls for innovative strategies including food fortification, while overcoming the drawbacks of taste and reactivity of iron. The aim of this work was to develop iron-rich colloidal microparticles laden with a Brazilian plant food extract from ora-pro-nobis (OPN, Pereskia aculeata Miller). We formulated electrostatic self-assembled complexes of oppositely charged chitosans and pectin laden with aqueous OPN extract. After characterisation of the physical properties, selected formulations were examined in their colloidal stability (50 mM NaCl, Dulbecco's modified eagle medium (DMEM), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)), production yield, iron association efficiency, transmission electron microscopy (TEM), cellular cytotoxicity and iron uptake using Caco-2 cells. The ζ-potential varied from ~ –25 mV to ~ +23 mV regardless of the degree of acetylation (DA) of chitosan. The production yield ranged between 20-26 %. The particles were stable at DMEM, SGF and SIF during 3 h. Iron association efficiency was ~60 % for systems charge ratio (n+/n–) = 5.00. TEM analyses revealed invariably spherical morphology. OPN-laden microparticles did not present cytotoxicity against Caco-2 cells. Higher cellular ferritin levels were determined for the particles comprising OPN extract and n+/n– = 5.00. We obtained in vitro proof of concept of the efficiency of chitosan/pectin particles to delivering iron from a Brazilian edible plant extract. The industrial potential of this approach as a viable alternative for iron fortification or supplementation by the food industry is yet to be realised.

Key-words: chitosan, pectin, ora-pro-nobis, microparticles, iron deficiency.

1. INTRODUCTION

Iron deficiency anaemia is the most frequent health problem in the world (Al Hassan, 2015; Soleimani & Abbaszadeh, 2011) being classified as the seventh wide reason of diseases, incapacity and deaths in the world (Hurrell et al., 2004). Effective measures are needed to implement iron food fortification programmes. However, iron confers a metallic taste to food, induces adverse reactions such as oxidation of lipids, and if not fully absorbed, promotes an accumulation in the gastric lumen and consequently irritation of the mucosa (Saha, Pandhi, Gopalan, Malhotra, & Saha, 2007; Schümann, Ettle, Szegner, Elsenhans, & Solomons, 2007). Iron is an essential mineral for the human organism due to its requirements for several metabolic functions such as oxygen transport, drug metabolism, steroid synthesis, DNA synthesis, ATP production and electron transport (Crichton, 2001).

Colloidal particles loaded with iron could be used to address some of the shortcomings of iron salts in fortified foods and supplements. Nano- and microparticles can associate, protect, and subsequently release and favour the oral absorption of iron. Chitosan is a cationic polysaccharide with low toxicity, biodegradability, biocompatibility (Luo & Wang, 2014) and mucoadhesive properties (Menchicchi et al., 2014; George & Abraham, 2006). Pectin is a polyanionic polysaccharide with low toxicity and potential for biomedical application such as scaffolds, drug delivery, tissue engineering and gene therapy (Martins et al., 2018; Ribeiro et al., 2014; Nishijima, Iwai, Saito, Takida, & Matsue, 2009). Polyelectrolyte complexes can be formed using chitosan and pectin from the electrostatic interactions occurring between carboxylic acid groups of pectin and amino groups of chitosan (Maciel, Yoshida, & Franco, 2015). When these polymers are homogenised in aqueous solutions, the formation of a self- assembled electrostatic complex takes place with unique properties that differ from those of the original components (Chen et al., 2010). The interactions between these polymers have been exploited in pharmaceutical and biomedical research (Luo and Wang, 2014), to obtain films/membranes (Maciel et al., 2015; Meng et al., 2010), hydrogels (Berger, Reist, Mayer, Felt, & Gurny, 2004), micro/nanoparticles (Maciel, Yoshida, Pereira, Goycoolea, & Franco, 2017; Luo, Teng, Li, & Wang, 2015), scaffolds (Martins et al., 2018), gene delivery systems (Santos-Carballal et al., 2015) and to support bone tissue engineering (Mallick, Singh, Rastogi, & Srivastava, 2018).

Using the concept of self-assembly polyelectrolyte electrostatic complexation, in this work, microparticles of chitosan-pectin were developed and loaded with an iron-rich

aqueous extract from an edible indigenous Brazilian vegetable namely OPN (Pereskia aculeata Miller). OPN is a cactus found in American regions (southern of the United States (Florida) and southeast of Brazil) (Maciel, Yoshida, & Goycoolea, 2018; Gronner, Silva, & Maluf, 1999). OPN belongs to the Cactaceae family with scandent habits and easy cultivation in regions with temperature above 25 °C and intense solar luminosity. Consumption of OPN has been associated with the prevention of iron deficiency anaemia, osteoporosis, and constipation (Almeida & Correa, 2012). It is a non-conventional vegetable food regarded as a bush or weed that presents important nutritional value, especially high-quality proteins (17.4-28.4 %) with 85 % digestibility (Lima Junior et al., 2013) and minerals such as iron (~ 81-142 $\mu g\ g^{-1}$) and calcium (Oliveira, Wobeto, Zanuzo, & Severgnini, 2013; Takeiti, Antônio, Motta, Collares-Queiroz, & Park, 2009). Non-conventional indigenous plants are used as a food, but normally, they are not part of the daily diet and their consumption circumscribes to the regions where they grow. Usually, these plants are underexploited and remain almost unknown. Their sustainable exploitation could represent a source of income as well as the discovery of novel and biofunctional foods with demonstrated health benefits. Given the considerable iron contents of OPN leaves, it can be regarded as an attractive alternative plant food to tackle iron deficiency anaemia.

Relevant strategies to combat the deficiency in iron comprise the supplementation and fortification using different iron salts (Zimmermann & Hurrell, 2007). Iron absorption mainly occur in the upper part of the small intestine and it is regulated to some extent by physiological demand. Iron is present in the diet in two forms, either as haem (Fe^{2+}) and non-haem iron (Fe^{3+}) which determines the mechanisms and quantity of iron absorption in the human body (Andrews, 1999). In majority of diets, iron is present in the non-haem form. According to Crichton et al. (2002) around 20 % of the non-haem iron consumed is absorbed through the gut enterocyte and available to the systemic circulation. Physiological factors and dietary components such as carbonate, oxalate, phosphates and phytate could promote a decrease in the bioavailability of iron (Somsook et al., 2005).

Research using iron from natural sources for the development of supplement formulations for oral delivery is scarce. Since the XIX century until now, iron is mainly supplemented using ferrous iron-based formulations (synthetic form) due to the low cost (Martnez-Navarrete, Camacho, Martnez-Lahuerta, Martnez-Monzo, & Fito, 2002). However, ferrous sulphate is very reactive; when used as iron-based supplements, it may

promote adverse gastrointestinal alterations related to the iron non-fully absorbed (Saha et al., 2007; Schümann et al., 2007). Therefore, the benefits involving an iron supplementation therapy are harshly limited (Schümann et al., 2007).

The main aim of this work was to examine the formulation of chitosan/pectin based microparticles laden with an aqueous extract of OPN, taken as a rich natural source of non-haem iron. We evaluated the formation of microparticles between the two biopolymers and OPN, and characterised their main physicochemical properties, namely the hydrodynamic size, ζ-potential, morphology, production yield, stability in physiological media, as well as the bioiron association efficiency, cytotoxicity and uptake by Caco-2 cells.

2. MATERIALS AND METHODS

2.1 Materials

Chitosan samples, here designed as low degree of acetylation sample "L-DA" and high degree of acetylation sample "H-DA" with high purity were acquired from Heppe Medical Chitosan GmbH (Halle, Germany) and with batch numbers 212-290814-02 and 212-170614-01, respectively; pectin from citrus peel was donated by CPKelko (GENU® 105, lot LI03024, Brazil) with high degree of esterification (DE = 68.2 %, Maciel et al., 2017). OPN leaves used to prepare the extract were kindly donated by the owners of São Luís Farm (Conceição do Araguaia-PA, Brazil). The exsiccate of OPN leaves was deposited in ESALQ/USP Herbarium (Piracicaba, Brazil), generating the number ESA136618. The reagents employed presented analytical grade. Water (ultrapure MilliQ, 18.2 MΩ cm at 25 °C) was used to prepare overall solutions and reagents.

2.2 Methods

2.2.1. Evaluation of the DA

The DA of chitosan was calculated according Lavertu et al. (2003). The analysis was performed using ^{1}H-NMR spectroscopy (Bruker, DRX 500 model, Switzerland) at 70 °C. Briefly, powder samples of both chitosans (5.0 mg) were solubilised in 1 mL of hydrochloric acid (HCl, 37.0 %). Afterwards, samples were frozen during 24 h at -20 °C and subsequently lyophilized during 12 h. Afterwards, one mL of D_2O was added to tubes containing the lyophilized sample and subjected to the analysis. The DA (%) of chitosan was determined using the Equation 1:

$$DA\ (\%) = 100 - \left[\left[\frac{H_1D}{H_1D + \frac{H-Ac}{3}}\right] * 100\right] \qquad \text{Eq. 1}$$

where, H_1D is the integral of peak of proton H_1 of the deacetylated monomer and $H-Ac$ is the integral of peak of proton H_1 of the peak of the three protons of acetyl group.

2.2.2. Determination of molecular weight distribution of chitosan

The molecular weight distribution and the corresponding parameters (weight average molecular weight (Mw) and number average molecular weight (Mn)) of the two chitosan samples was carried out using an AF2000 Multiflow system (Postnova, Analytics, Germany) equipped with automatic sample injector (PN5300) and coupled to a MALS Detector, 21 angles (PN3621), refractive index (RI) detector (PN3150) and UV detector (PN3211, 280 nm and 220 nm wavelengths). The channel formed by a PTFE spacer between two walls was of trapezoidal geometry with 350 μm thickness and it was kept at 30 °C. A regenerated cellulose membrane (Z-AF4-MEM-612-1KD) with a molar mass cut-off of 1 kDa was used as accumulation wall. Due to the cationic nature of chitosan, and in order to minimise the interactions with the membrane, a solution of diluted acetate buffer (0.18 M acetic acid/ 0.02 M sodium acetate pH 3.7) was used as the carrier liquid. This solution charged the membrane positively ensuring the elution of the polymer. Before measurements, all samples were filtered through membrane with 5 μm (EMD Millipore, USA). For the experiments, the detector flow rate was set to 0.5 mL min^{-1} for all samples and 50 μL volume of sample (2 mg mL^{-1}) was injected at a rate of 0.20 mL min^{-1} for an injection period of 6 min and cross flow (CF) set at 3 mL min^{-1}. After a focusing period of 3.30 mL min^{-1} and a transition period of 0.2 min, the profile of the crossflow was gradually decreased in 60 min through a series of consecutive steps as follows: 1) For 0.2 min, the CF was kept constant at 3 mL min^{-1}; 2) CF was then decreased at an exponent of decay of 0.40 to 0.22 mL min^{-1} over 30 min period; 3) CF was further decreased to 0.11 mL min^{-1} during 5 min at 0.80 exponent decay; 4) CF was finally decreased to 0.06 mL min^{-1} at 0.80 power decay over 5 min after which 5) CF was kept at flow 0.06 mL min^{-1} for additional 20 min. Data and collection analysis were performed with NovaFFF version 2.0.9.9. The measurements were conducted in triplicate per sample and a blank (acetate buffer carrier liquid pH 3.7) was also run. All calculations were performed on the subtracted detector signals (sample minus blank signals). The RI signal

was used for M_w calculations using an average refractive index increment (dn/dc) for chitosan of 0.19 (Nguyen, Winnik & Buschmann, 2009). Data were fitted to a Zimm model.

2.2.3 Preparation of microparticles using OPN extract

2.2.3.1 Chitosan solution

Chitosan was used as received and prepared according to Maciel et al. (2017). It was dissolved (5.0 mg mL^{-1}, w/w) in sodium chloride solution (50 mM NaCl) with 5 % stoichiometric excess of HCl by constant magnetic stirring (25 ± 1 °C, 14 h). Afterwards the solution was filtered using EMD Millipore membranes (5.0 µm, USA).

2.2.3.2 Pectin solution

Pectin was used in the purified form. It was dissolved (5.0 mg mL^{-1}, w/w) directly in OPN extract (section 2.2.3.3) and kept under constant magnetic stirring (50 ± 1 °C, 1 h, to assure the complete solubilisation of the pectin) and followed at 25 ± 1 °C during 13 h. The pH of the solution was not adjusted. Membranes (5.0 µm, EMD Millipore, USA) were used to filter the pectin solution.

The pectin purification was conducted according to the protocol described by Bernabé, Peniche, & Argüelles-Monal (2005). Pectin (2.0 g L^{-1}) was solubilised in a solution of sodium chloride (50 mM NaCl) by vigorous and constant magnetic stirring at 50 ± 1 °C for 1 h and followed during 13 h at 25 ± 1 °C. The sample was filtered sequentially using sintered glass filters with four different pore diameters (80.0, 60.0, 40.0 and < 10.0 µm) and five membranes with pore diameters (0.20, 0.45, 0.80, 1.20 and 5.00 µm, EMD Millipore, USA). Ethanol was added gradually up to a final concentration (80.0 %) to promote the pectin precipitation. The precipitate was removed using a centrifuge (Sorvall, R-5 plus model, USA) at 7000 rpm and 10 °C during 30 min. Resultant solid material was carefully washed out using different ratios of ethanol/water (100/0, 90/10, 80/20 and 70/30) during 5 min. Purified pectin was kept in an oven with air circulation (Tecnal, TE-394/1 model, Brazil) at 25 ± 1 °C for 48 h to evaporate the residual ethanol.

2.2.3.3 OPN extraction and characterisation

The methodology used to prepare the aqueous OPN extract was adapted from the previous work by Lima Junior et al. (2013). Briefly, OPN dried leaves (1 g) were dispersed in 100 mL of sodium chloride (50 mM NaCl) and maintained in magnetic

stirring. The temperature and time were 75 °C for 1 h, established in preliminary experiments. Two filtration steps were performed: the first using Buchner filters (sizes 1, 2, 3 and 4) and the second with membranes of different pore sizes – 5.0 μm (Química Moderna, cellulose nitrate filters, Brazil) and 0.8 μm (Química Moderna, ester mixing membrane filters, Brazil).

Mineral characterisation of OPN extract: copper, iron, magnesium, and zinc were quantified by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Scientific, iCAPQc model, Germany); and calcium, manganese, phosphorous, potassium and sulphur were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) (Thermo Scientific, iCAP 7400 Radial model, Germany). Samples were all diluted ten-fold using 2.0 % HNO_3 before analysis. The standard phenanthroline method (SMEWW, 1999) was used to determine the total iron and ferrous iron (Fe^{2+}) in the OPN extract. Ferric iron (Fe^{3+}) was calculated through subtraction from total iron and ferrous iron. A standard curve using ferrous ammonium sulphate (0, 25, 50, 75, 100, 125, 150, 175 and 200 μg of iron in 50 mL of solvent) was generated and samples diluted accordingly.

2.2.3.4 Production yield of microparticles

Particles were produced using electrostatic self-assembling method according to the protocol described in our previous study (Fuenzalida et al. 2014). Briefly, chitosan-pectin microparticles systems comprising OPN extract were obtained considering different equivalent charge ratio (n+/n–) namely 0.10, 0.25, 0.50, 0.75, 1.00, 2.00, 4.00, and 5.00, and total equivalent charge ($n^+ + n^-$) of 1.0×10^{-6}. The total charge was also evaluated 2.0×10^{-6} and 3.0×10^{-6}, but the results were not good to form microparticles, due to the formation of agglomerates in these systems. To screen the formation of complexes of varying equivalent charge ratio, the stock solutions of chitosan and pectin/OPN were mixed in a 96-well microtiter plate (Sarstedt, Germany). Different volume aliquots of chitosan solution (5.0 mg mL^{-1}, w/v) were placed first into the microwells, to which varying volume aliquots of pectin solution (5.0 mg mL^{-1}, w/w) prepared in OPN extract were dispensed and thoroughly mixed by flushing in and out of the pipette tip. A first screening enabled to discern between mixtures of clear/limpid appearance from slightly turbid ones. Two optimal formulations were selected for the next experiments, namely one with excess of pectin (n+/n– = 0.25) and the other containing chitosan in excess (n+/n– = 5.0), with negative and positive ζ-potential, respectively (Section 2.2.4).

For iron uptake experiments, the microparticles were prepared following the same protocol described above but replacing the OPN extract by $FeSO_4$ solution. The objective was to compare the particles containing different type and source of iron.

Isolated microparticles: whenever necessary, it was carried out by centrifugation (40 min, 12000 × g and 20 °C) using tubes with glycerol (15 µL). The pellets formed were resuspended in 50 mM NaCl or Dulbecco's modified eagle medium (DMEM, 100 µL), depending on the further analysis. Microparticles were prepared in a laminar flow chamber under adequate microbiological conditions.

2.2.4 ζ-potential and particles size distribution

The ζ-potential was measured by mixed-mode phase analysis light scattering (M3-PALS). The particle size distribution was measured by dynamic light scattering coupled with non-invasive back scattering (DLS-NIBS) at a scattering angle of 173° with automatic gain. Both measurements were carried out using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a red laser light output (λ= 632.8 nm). Systems with charge ratios (n+/n–) 0.25 and 5.00 total charge 1.0×10^{-6} and sodium chloride 50 mM (Section 2.2.3.4). All measurements were carried out in triplicate at 25 ± 0.2 °C.

2.2.5 Production yield of microparticles

Microparticles elaborated with OPN extract were centrifuged (40 min, 12000 × g and 20 °C). The resultant pellet (after removing the supernatant) was frozen at -20 °C followed by the lyophilisation process for 24 h. The production yield of the particles was determined considering the chitosan, pectin (solubilised in OPN extract) and 50 mM NaCl masses used to prepare the initial solution and the mass of the pellet formed, according below:

$$Production\ yield\ (\%) = \frac{M_{pellet}}{(M_c + M_p + M_{NaCl})} * 100 \qquad \text{Eq. 2}$$

where, M_{pellet} is the mass of lyophilised particles (mg) containing OPN extract, M_c is the mass of chitosan (mg), M_p is the mass of pectin (mg) added of OPN extract or $FeSO_4$ solution and M_{NaCl} mass of NaCl (mg) used in the microparticles formation, respectively.

2.2.6 Stability of microparticles

The stability of the systems was analysed according to the protocol of Trapani et al. (2013). Briefly, an aliquot (50 µL) of microparticles isolated solution was incubated in a microtiter plate incubator (Heidolph, Titramax model, Germany) at 37 °C using cuvettes containing 1.0 mL of 50 mM NaCl, DMEM, SGF or SIF. SGF and SIF were prepared according with the United State Pharmacopeia 42 – National Formulary 37 (USP-NF, 2019). The particle size was measured at times 0, 20, 40, 60, 120 and 180 min by DLS-NIBS (Section 2.2.4). Triplicate measurements were performed.

2.2.7 TEM analyses

The analyses were performed using JEM-1400 TEM (JEOL, Peabody, MA, USA) operated at 100 kV to verify the microparticles with or without OPN extract prepared with chitosan H-DA at values of charge ratios ($n+/n-$) 0.25 and 5.00). Equal amounts of fresh samples were homogenised with uranyl acetate solution (negative staining, 1 %, w/v). Samples (8 µL) were placed onto a copper grid covered with Formvar® film and the excess of liquid was removed with the aid of the filter paper.

2.2.8 Determination of iron association efficiency in microparticles

The determination of iron association efficiency (from OPN extract and $FeSO_4$ solution) in microparticles was determined using centrifugation process of the particles. Then, the iron present in the supernatant was quantified. Microparticles samples were centrifuged (40 min, 12000 × g and 20 °C). The iron quantity not associated was determined by ICP-MS in aliquots of supernatant (see section 2.2.3.3). A standard curve was used to determine the iron content. The data were the average of three independent experiments. The iron association efficiency in microparticles was determined by Equation 3 (Zariwala et al., 2013a):

$$Iron\ association\ efficiency\ (\%) = \left[\frac{(T_i - F_i)}{T_i}\right] * 100 \qquad \text{Eq. 3}$$

where T_i is the total iron quantity added at the formulation and F_i is the unincorporated iron determined in the supernatant.

2.2.9 Iron availability studies using Caco-2 cell model

2.2.9.1 Cell cultivation and cell viability (MTT assay)

The Caco-2 cell line (HTB-37^{TM}) was acquired from American Type Culture Collection (Rockville, MD, USA). The cells were cultivated in high glucose DMEM GlutaMAX^{TM} medium, supplemented with foetal bovine serum (10 %) and penicillin-streptomycin (1 %) under standard conditions (37 °C and 5 % CO_2). Cytotoxicity of the particles and pure OPN extract was evaluated using MTT assay (Gursoy, Garrigue, Razafindratsita, Lambert, & Benita, 2003). Briefly, confluent cells in 96 well plates were incubated with OPN extract diluted in DMEM to obtain different concentrations of iron (92.5, 185.0, 222.5, 445.0 and 890.0 ng mL^{-1}) relating to the maximum content of iron present in the particles and linked to the quantity of pectin solution used to prepare the charge ratios (n+/n–) with excess of pectin (0.25) and excess of chitosan (5.00).

Microparticles were prepared at different concentrations of iron from diluted OPN extract, charge ratios (n+/n–) 0.25 and 5.00 and using chitosan with DA 22.3 %. Isolated particles were dispersed in cell culture medium (DMEM, 100 µL), incubated at 37 °C during 4 h and controlled atmosphere (5 % CO_2). Afterwards, the samples were removed and replaced by DMEM. In each well 25 µL of MTT solution (5 mg mL^{-1}, prepared in PBS) was added. After 4 h of incubation the medium was aspirated, and DMSO was added to bleach out the dye. Absorbance was measured at 570 nm using a microplate reader (Tecan, Spark 10M model, Austria). Positive (DMEM without particles) and negative (induced cell death using 4 % triton X) controls were used (Gursoy et al., 2003). Each experiment was performed in three different cells passages with eight parallel wells.

2.2.9.2 Iron uptake into Caco-2 cells

Iron uptake was determined as cellular ferritin content (Zariwala et al., 2013a). Briefly, Caco-2 cells were differentiated over 14 days with DMEM medium changes every two days. Then, DMEM medium was suctioned and the cells washed followed by the incubation in serum-free MEM (modified Eagle's medium) for 24 hours. The following day, Caco-2 cells were washed using buffer solution (three times). The microparticles containing iron combined with aliquots of test media MEM (pH 5.8, simulating the pH of duodenum) were buffered with 10 mM 2-[N-Morpholino] ethane-sulfonic acid. Cells were placed in an incubator at 37 °C during 2 h under agitation (25 rpm) according to Zariwala et al. (2013a). Afterwards, the media containing the treatments were aspirated and the cells washed three times in a sequence: wash solution was used twice and another one using a surface bound iron removal solution. According to Glahn et al. (1995), the addition of 5 µM of sodium hydrosulphite and 1 µM of

bathophenanthroline disulfonate is necessary and efficient to remove the iron unabsorbed present in the cells surface. After the wash process, MEM was added into Caco-2 cells and placed in an incubator (37 °C during 24 h). Considering the effect of different iron sources (OPN extract and $FeSO_4$ solution) on iron uptake, parallel experiments with aqueous OPN extract and $FeSO_4$ solution containing equal iron concentrations (1522 ng mL^{-1}) were carried out. After 24 h incubation, the medium was removed and cells washed with wash solution (twice). Different amounts of $FeSO_4$ solution (5, 10, 20, 50 and 80 µM) were used to evaluate a dose-response pattern. The incubation time for the iron uptake process was set to 2 h, based on previous studies performed by Sharp (2005). The cells were harvested using a CelLytic lysis buffer (Sigma-Aldrich) including protease inhibitor cocktail (Sigma-Aldrich, UK) and lysed using a plate shaker at 4 °C, 15 min and 50 rpm. Cells were scraped, placed into a 1.5 mL microcentrifuge tube and centrifuged for 15 min (14,000 g, 4 °C). The supernatants (whole cell lysate) were stored at −20 °C. Uptake experiments were performed in three independent experiments.

2.2.9.3 Ferritin measurement in cell lysates

Ferritin content was determined by ELISA kit (Ramco Laboratories, UK) according to the methodology proposed by Zariwala, Somavarapu, Farnaud, & Renshaw (2013b). A standard curve was used covering a range of 0, 6-200 ng standard mL^{-1}. Ferritin values were normalised to total cell protein (ng ferritin/mg protein). BCA assay (Pierce, Thermo Fisher Scientific, UK) was used to determine the total cell protein content.

2.3. Statistical analysis

The software Statistic (version 7.0, Statistic Inc., USA) was used to perform the statistical analysis. Differences between the averages were identified by ANOVA and Tukey' test ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Chitosan characterisation

The characteristics of the used chitosan samples were as follows. L-DA: DA 8.6 %, Mw $4.12 \pm 0.30 \times 10^4$ g mol^{-1}, Mn $2.52 \pm 0.30 \times 10^4$ g mol^{-1}, polydispersity index (PDI, =Mw/Mn) 1.6 ± 0.01; H-DA: DA 22.3 %, Mw $3.22 \pm 3.7 \times 10^4$ g mol^{-1}, Mn $2.02 \pm 0.30 \times 10^4$ g mol^{-1}, PDI (=Mw/Mn) 1.6 ± 0.14. These characteristics confirmed that the two chitosan samples differed only in the DA by almost 3-fold, and hardly at all on the

molecular weight parameters. All these parameters are known to be important in the formation and physicochemical characteristics of microparticles (Kleine-Brüggeney, Zorzi, El-Gueddari, Moerschbacher, & Goycoolea, 2015; Kumar & Ahuja, 2013). The Mw of the employed chitosans could be considered a medium range. Chitosans of this type have been found amenable for the formation of nanoparticles by ionotropic or by reverse emulsion gelation (Goycoolea et al. 2016).

3.2 Mineral characterisation of OPN extract

Results of mineral determination in OPN extract (Table 1) indicate a major concentration of potassium and magnesium, followed by sulphur, phosphorous and calcium. A significant quantity of total iron (2030 ± 105 µg L^{-1}) was analysed demonstrating its efficient extraction from the OPN dry leaves. As expected for plants (vegetable origin), the quantity of ferric iron (1370 ± 107 µg L^{-1}) was twice as high as compared to ferrous iron (660 ± 44 µg L^{-1}). Our results differ compared to those reported by Takeiti et al. (2009) and Lima Junior et al. (2013) in fresh OPN leaves and OPN extract, respectively, which could have related to external factors (such as climate and local growth conditions), harvesting season and pre-processing methods.

3.3 ζ-Potential and particle size distribution analysis

The particle size and ζ-potential are relevant physicochemical parameters known to have a direct impact on the stability, biodistribution, process of absorption, cellular uptake and overall in vivo performance of different types of microparticles. They could also affect the drug loading capacity as well as the in vitro and in vivo drug release properties.

The ζ-potential behaviour and the pH range to form the systems involving microparticles were defined in a previous work published from our group (Maciel et al., 2017). At different total charges (n+ + n−) of 2.0×10^{-6} M and 3.0×10^{-6} M, aggregation inexorably occurred for all tested charge ratios (n+/n−) tested. By contrast, at 1.0×10^{-6} M, colloidal particles were formed when blending chitosan L-DA (DA 8.6 %) and pectin at the charge ratios (n+/n−) 0.10 and 0.25 (excess of pectin), and 2.00, 4.00 and 5.00 (excess of chitosan). Whereas with chitosan H-DA (DA 22.3 %) at the same total charge, the formation of particles was evidenced throughout all the tested charge ratios (n+/n−). Based on these preliminary observations, new tests were carried out under identical conditions (varying n+/n- charge ratios, total charge 1.0×10^{-6} M and with both chitosans)

to confirm the formation of particles and measure the ζ-potential and particles size.

It was expected that carboxylate groups of pectin (i.e. bearing negative charge) would be interacting electrostatically with amino groups from chitosan (i.e. bearing positive charge) to obtain particles under controlled conditions by polyelectrolyte complexation. Particles size below ~2600 nm (microparticles) were found for complexes at charge ratios (n+/n−) of 0.10, 0.25, 0.50, 0.75, 1.00 and 5.00 for chitosan withal DA 8.6 % (Figure 1a). The PDI varied in the range 0.12 ± 0.04 and 0.33 ± 0.07, in good agreement with published values for chitosan-based nanoparticles (Goycoolea et al., 2016; Kleine-Brüggeney et al., 2015). For the ζ-potential values (Figure 1a), the charge ratios (n+/n−) below 1.00 showed negative values (~ −25 to ~ −19), thus confirming the excess of pectin charges in this complex system. Considering the charge ratios (n +/n−) above 1.00, the resultant charge of the system increased exhibiting a positive ζ-potential, the expected consequence of the charge excess of chitosan. A resemblance among the results obtained for the systems using different chitosans was observed (DA 8.6 % and DA 22.3 %). Particles with average size lower than ~1500 mm and negative ζ-potential were found at charge ratio (n+/n−) below 1.00 (Figure 1), as a result of the surplus of negatively charged pectin. At n+/n− > 1.00, the ζ-potential of both systems reversed to positive in the systems comprised by both type of chitosans. The results provide unequivocal evidence that the colloidal particles are formed as the result of polyelectrolyte complexation driven by charge compensation. In support of this, note that at charge ratio (n+/n−) near to the stoichiometric value, (between charge ratio (n+/n−) ~1.0 and ~2.0), the ζ-potential attains a neutral value. Our results agree with previous studies using chitosan (DA ~20 %; Mw ~2.3 × 10^5 g·mol^{-1})/polyguluronate polyelectrolyte complexes (Argüelles-Monal, Cabrera, Peniche, & Rinaudo, 2000) and chitosan/pectin/insulin nano- and microparticles (Maciel et al., 2017). The complexation of chitin and pectin has also been studied in more recent studies using ζ-potential as one of the main experimental technique (Kulikouskaya, Lazaouskaya, & Agabekov, 2019; Niu et al., 2019). Considering the above results, two systems were selected for each type of chitosan (L-DA = 8.6 % and H-DA = 22.3 %) for further studies: one involving a surplus of pectin (n+/n− = 0.25) and other using surplus of chitosan (n+/n− = 5.00). The total charge was kept at 1.0 × 10^{-6} M.

3.4 Production yield of microparticles

The production yields of iron-loaded microparticles (Table 2) were similar in all formulations studied, except for the chitosan DA 8.6 % and charge ratio (n+/n−) 0.25. However, within experimental error the size data for the various formulations were of the same order of magnitude. Independently of chitosan DA and charge ratio (n+/n−) studied, the production yield was kept between 20-26 %. The relatively low yield is the consequence of the incomplete incorporation of the formulation components in the formed complexes during the preparation and their loss to the supernatant, thus reducing the production yield. Iannone et al. (2017) evaluated the production yield of grape seed extract-loaded chitosan micro-particles with different concentration of extract in the system and obtained values between 42.3 and 64.8 %. In general, the results obtained for yield production were considered in overall good agreement with reported values on previous studies on chitosan microparticles for different drug delivery systems, namely 12-48 % for tea polyphenol-Zn complex (Zhang & Zhao, 2015), 24-84 % for vancomycin (Cerchiara et al., 2015), 30-46 % for antituberculotic drugs (Oliveira et al., 2017) and 33-58 % for vitamin B12 (Carlan, Estevinho, & Rocha, 2017).

3.5 Stability of microparticles

The evolution of the Z-average particle hydrodynamic diameter during incubation in four different environments, namely 50 mM NaCl, cell culture medium DMEM and simulated gastrointestinal fluids (SGF and SIF) is shown in the various panels of Figure 2. First, we evaluated the stability in 50 mM NaCl (i.e., the same solvent condition in which the particles were originally formed), aimed as a control to the rest of the experiments (Figure 2a). At time zero, the particle size was essentially the same as that of the freshly prepared formulations (cf., Figure 1 and Figure 2a). A closer inspection of the plot reveals that for systems comprising chitosans of DA 8.6 and 22.3 % and charge ratio (n+/n−) 5.00 there was an increase from 2.2 to 4.1 µm and from 4.7 to 6.1 µm, respectively, though no visible aggregation was observed. Conversely, systems comprising both chitosans and charge ratio (n+/n−) 0.25, attained noticeably smaller sizes (~1 µm) and remained essentially unchanged during the course of the assay. Second, the systems incubated in DMEM showed overall smaller initial particle size (~380 – ~550 nm) than in 50 mM NaCl (Figure 2b). Systems comprising chitosan of DA 22.3 % and high charge ratio (n+/n−= 5.00) were the largest and increased from ~550 to ~900 nm, while the corresponding systems of low charge ratio (n+/n−= 0.25), increased from ~425 to ~800 nm over the course of the assay. Interestingly, particles comprising chitosan DA and 8.6 % and high charge ratio (n+/n−=

5.00) remained stable up to 60 min (~500 nm), and beyond this time, increased to ~775 nm. In turn, particles comprising chitosan DA and 22.3 % and low charge ratio (n+/n−= 0.25), showed only a slight increase in particle size from ~375 nm to ~450 nm. Third, in SGF (Figure 2c), the particles also attained sub-micron sizes invariably smaller than in 50 mM NaCl and were consistently smaller for systems of charge ratio n+/n− 5.00 than 0.25 (~250 vs. ~400 nm), independent of chitosan's DA. Finally, in SIF (Figure 2d), the particles attained sizes that varied from ~750 nm to ~3500 nm for systems of charge ratio (n+/n−) 0.25 and 5.0, respectively. In all the systems, except those comprising chitosan DA and 8.6 % and high charge ratio (n+/n−= 5.00), the particles size remained unchanged during incubation. For such system, an unusual behaviour was observed, in which the size initially increased from ~1250 to ~2500 nm and after 3 h it decreased back to the original value, thus describing a bell-shaped curve.

The colloidal stability against varying conditions of pH, ionic strength and simulated physiological conditions (e.g. during digestion) can largely affect their cellular uptake effective delivery of the payload. Often, the colloidal stability of microparticles is assessed only in PBS (pH 7.4) as a surrogate of plasma and other biological fluids (Soliman, Zhang, Merle, Cerruti, & Barralet, 2014). In previous studies (Goycoolea et al. 2012), we have evaluated the stability of chitosan-based nanocapsules coated with chitosans of different DA in RPMI and MEM cell culture media and found that in general, the nanocapsules comprising chitosans of high DA and low M_w were the most stable. The role of the chitosan at the surface on the hydration in the presence of different ions has been crucial to explain the colloidal stability of these systems (Santander-Ortega, Peula-García, Goycoolea, & Ortega-Vinuesa, 2011). In our study, we were interested in evaluating the stability in cell culture medium (DMEM) as well as in simulated gastrointestinal fluids as these are relevant for in vitro cell culture studies and for the potential development of a formulation for oral delivery. As a control, we assessed the evolution of the particle size in NaCl 50 mM, the same solvent used to prepare the particles. Even when the particles comprising an excess of chitosan (n+/n- =5.0) experienced a noticeable growth in their size, we were confident that they did not aggregate. It was interesting noting that in both DMEM cell culture medium and SGF, the particles attained a smaller size than that in 50 mM NaCl. We attribute these differences to the varying ionic strength and pH conditions from the originally used to produce the particles. Given that the particles form spontaneously by polyelectrolyte electrostatic self-assembly upon mixing, they are known to be kinetically trapped in their

493 conformation and size (Costalat, David, & Delair, 2014). Any subsequent change in the
494 state of charge ionisation (i.e., driven by pH or ionic strength) in chitosan and pectin
495 polyelectrolytes, may lead to a weakening of the complex and re-structural
496 rearrangements that can result in either compaction or expansion of the originally formed
497 complexes, as they attain a thermodynamically more stable state. These effects are
498 particularly noticeable in Figure 2c and 2d, showing the evolution of the size upon
499 incubation in SGF (pH 1.2) and SIF (pH 6.8), respectively. In SGF, the particles with an
500 excess of chitosan (n+/n- = 5.0), attain half as smaller size than those bearing an excess
501 of pectin (n+/n- = 0.25). By contrast, in SIF (pH 6.8), the carboxylate groups of pectin
502 are bound to be fully ionised while the amino ones in chitosans are bound to be
503 predominantly neutral, hence, the particles bearing an excess of pectin attained the
504 smaller size. According to Vaarum & Smidsrod (2005), at pH around 7.0 (e.g. as in SIF),
505 chitosan is present in non-ionised form, which could explain the non-stability of the
506 particles containing surplus chitosan. By contrast, the compaction in size from the original
507 condition observed in SGF, can be attributed to a reduced aggregation, the consequence
508 of overall greater charge density, particularly for the particles comprising a surplus of
509 chitosan (n+/n- = 5.0). In studies developed by Huang et al. (2019) in zein/pectin core-
510 shell nanoparticles, it was evaluated the influence of the ionic strength (0-70 mM) on
511 particle stability. They observed an increase on particle size when the level of salt was
512 increased from 0 to 50 mM, which may be due to a weakening of the electrostatic
513 attraction between the polymers used to form the microparticles. It is known that steric
514 and electrostatic repulsion, as well as weakening of hydrophobic attraction favour
515 colloidal stability.

516 These results of our study agree closely with our previous work (Maciel et al. 2017),
517 in which we used similar chitosan/pectin microparticles loaded with insulin. Indeed, in
518 the previous study, we also observed overall colloidal stability in cell culture medium
519 MEM SIF and SGF media. Also, our results confirmed the overall decrease in the particle
520 size upon incubation in cell culture media and in SIF. Other studies, for example, Zhang,
521 Wang, Ni, Zhang, & Shi (2016) produced nanoparticles based on chitosan and poly(2-
522 acry1amido-2-methylpropanesulfonic acid) by electrostatic interaction varying the
523 weight ratio of the constituents, structure and properties. They observed that smaller
524 particles were generally more stable when compared to larger ones after exposition to
525 physiological conditions. Andreani et al. (2015) verified that smaller particles tend to be
526 more effective to enhance the process of absorption in the intestinal epithelium. Bagre,

Jain, & Jain (2013) prepared chitosan nanoparticles coated with alginate loaded with enoxaparin and reported their low stability in SIF conditions (pH 7.4). They attributed this to chitosan (Mw 150 kDa, DA 85 %, purified viscosity grade 80 cps). Chen et al. (2009) evaluated the stability of the oral heparin delivery systems based on chitosan nanoparticles. They observed stable systems under acid condition (pH 1.2) and attributed this behaviour to the electrostatic interaction existing amid the ionized form of chitosan and the bioactive payload (heparin). It was also verified that heparin began to release from the systems with the increase of the pH to 6.6 or 7.0, as a resulting of the swelling process. At pH 7.4, the release of heparin was attributed to the nanoparticles disintegration. These results suggested that the nanoparticle stability in neutral and slightly basic pH (physiological condition of intestine) decreases, promoting the release of heparin. Yuan, Jacquier, & O'Riordan (2018) produced chitosan-polyphosphoric acid beads to entrap different bioactive (bovine serum albumin, insulin, casein hydrolysate and whey protein isolate) and showed stability for the systems in SGF. In SIF, the stability was dependent of the protein type, with best results for insulin and whey protein isolate.

3.6 Determination of iron association efficiency at microparticles

Iron association efficiency was determined considering two sources of this mineral: OPN extract and $FeSO_4$ solution with the objective to evaluate whether different sources (plant and synthetic) would differ in their ability to uptake iron into Caco-2 cells. Furthermore, it was necessary to determine the iron association efficiency of the systems for calculation of iron uptake. The association efficiency of iron from two different sources (OPN extract and $FeSO_4$ solution) into particles considering charge ratios (n+/n−) 0.25 and 5.00 and chitosan H-DA (22.3 %) are shown in Table 3. The maximum amount of iron for the charge ratio 0.25 was 890 ng mL^{-1}, while for the charge ratio 5.00 was 185 ng mL^{-1}. Iron association efficiency (45.78 %) was achieved for the systems prepared with $FeSO_4$ solution and charge ratio (n+/n−) 0.25 than those prepared with OPN extract. The association efficiency increased (59-63 %) for overall systems prepared at charge ratio (n+/n−) 5.00, considering the different iron source. It suggests that a surplus of chitosan could substantially influence the association efficiency of the iron rich-OPN into microparticles. Moreover, it is worth noting that carboxylate groups from pectin could also complex iron in the microparticulate proposed system.

Model nanoparticulate systems have an elevated association or encapsulation efficiency (Bayat et al., 2008). Electrostatic interactions between carboxylate groups of

anionic polymers (i.e., pectin) and amino functions from chitosan have considerable influence in the association efficiency of bioactive in chitosan nanoparticles (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997). It is possible to notice clearly from our results, that the resultant iron association was related to the proportional quantity of chitosan in the system. Hermida, Roig, Bregni, Sabés-Xamaní, & Barnadas-Rodríguez (2010) and Xia & Xu (2005) evaluating the use of chitosan in liposome systems observed that the presence of this polymer promoted an improvement on iron loading. Bhatia & Ravi (2003) proposed that a steady complex could be formed between iron and chitosan, promoting better incorporation. This observation would explain the high association efficiency observed in charge ratio (n+/n−) 5.00 (chitosan in excess). The fact that the production yield results were practically the same for overall formulations studied seems to be in accordance with the proposal that iron could be complexed in the system formed between chitosan and pectin. Further insight is needed to deepen our understanding of how iron interacts with the microparticles. This could indeed be addressed in future studies using FTIR, DSC, as well as other techniques such as AF4 coupled to ICP-OES. Whilst there is a lack of comparable studies investigating Fe association efficiency in chitosan-pectin particles, encapsulation of carotenoids into chitosan microparticles resulted in over 95 % efficiency (Rutz, Borges, Zambiazi, da Rosa, & da Silva, 2016) and Alencaste et al. (2006) encapsulating vitamin E using chitosan/carboxymethylcellulose reported an efficiency of 81 %. Loading iron on solid lipid nanoparticles using stearic acid by double emulsion solvent evaporation process resulted in iron incorporation efficiency in the range of 48-87 % (Zariwala et al. 2013a).

3.7 TEM analyses

Micrographs tending to present, in the most of cases, spherical shape (highlighted with arrows in the figures), were recorded for systems using both chitosans and charge ratio (n+/n−) indicating that the different DA studied would not be influencing the formation process of the particles (Figures 3). In the particles loaded with iron using chitosan DA 22.3 % it was relevant to verify that the systems exhibited similar spherical shape than the corresponding blank samples (Maciel et al. 2017). Similar results were found in the literature regarding systems involving natural compounds-loaded into the particles. For instance, Belscak-Cvitanovic et al. (2015) produced micro-particles of alginate-protein coated with chitosan or pectin to delivery bioactive compounds (flavan-3-ol antioxidants and caffeine) found in green tea extract and obtained microbeads spherically shaped;

Iannone et al. (2017) studied the formation of chitosan microparticles to entrap grape seed extract and observed spherical shape of the microsystems; Ge, Yue, Chi, Liang, & Gao (2018) evaluated nanocomplexes of chitosan hydrochloride and carboxymethyl chitosan loaded-anthocyanin (natural pigment) and found spherical structure for the system; among other. Indeed, this is a topic that is receiving increasing traction.

3.8 Evaluation of particle cytotoxicity

The cytotoxicity experiments employing Caco-2 cells could be used as a preliminary information to further studies indicating the potential toxicity on the intestinal tissue and providing suitable concentrations in a permeability study that involves any new bioactive compounds (Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, & Chowwanapoonpohn, 2007). In vitro cytotoxicity of OPN extract free of particles and iron-loaded microparticles was performed (Figure 4) at different concentrations of loaded iron (92.5, 185.0, 222.5, 445.0 and 890.0 ng mL^{-1}). These concentrations are reflecting the maximum content of iron present in the final particles. As we previously documented (Maciel et al. 2017), blank particles of chitosan-pectin did not lower the cell viability values below ~90 % even when applied at concentration 100 µg/cm^2 and 4 h incubation time. Likewise, OPN extract alone (Figure 4a) did not affect cell viability within the dose range studied (92.5-890.0 ng mL^{-1}). This was an essential point to test before considering the properties of the OPN extract as a potential source of iron supplementation.

When OPN extract was encapsulated by the chitosan-pectin microparticles, the cell viability remained above ~80 % in all formulations (Figure 4b). An investigation focused on the bioactivities properties, purification process, conditions of OPN cultivation could provide an useful information for further studies. Loretz & Bernköp-Schnurch (2007) and our own studies (Maciel et al. 2017) have evaluated nano- and microparticles systems containing chitosan and observed a decrease in Caco-2 cell viability. The authors suggested that it would be related to the electrostatic interactions existent among the cell membrane and polymeric nanoparticles. Zhang & Zhao (2015) produced nanoparticles using chitosan and obtained upward ~84.0 % of cell viability. Sharma, Shree, Arora, & Kapila (2017) evaluated a lactose-iron complex and observed lower cytotoxicity for this system compared to ferrous sulphate solution.

3.9 Iron uptake into Caco-2 cells

According to Hu et al. (2019), natural polymer-based colloidal particles could be

administered by the oral route to deliver bioactive compounds. In this study, we addressed the efficacy of chitosan/pectin microparticles to deliver iron intracellularly. To this end we adopted an in vitro assay with intestinal Caco-2 cells. This cell line is an accepted model system to determine cellular iron uptake with ferritin induction frequently used as a readout (Zariwala et al., 2013a). Cell models such as Caco-2 cells, present various advantages related to their facility and reproducibility that permit the comparison of the results inter-laboratory easily (Lea, 2015).

The iron uptake by Caco-2 cells from chitosan-pectin microparticles from the two different sources of iron examined (OPN extract and $FeSO_4$ solution) was evaluated over a period of two hours, in line with previous studies (Zariwala et al., 2013b). The ferritin formation was determined after further two hours, and compared to iron available in OPN extract and $FeSO_4$ solution free of particles (Table 4). Of note, the excess of chitosan in the microparticle systems (n+/n- ratio 5.0) loaded with iron-rich OPN extract promoted an ~two-fold increase in cellular iron uptake, from 3.53 ± 0.21 to 6.81 ± 0.36 ferritin ng mg^{-1} of cell protein. By contrast, when OPN extract was replaced by $FeSO_4$ solution as a source of iron in the formulations, the cells showed an overall greater absorption of ferritin. These results were expected considering the higher availability of ferrous iron (in $FeSO_4$ solution) in comparison to plant iron in OPN extract, where ~67 % is ferric iron. Interestingly, we observed that for microparticles of n+/n- ratio 5.0 loaded with OPN extract, the excess of chitosan resulted in an increase of iron association efficiency and uptake with respect to the particles with an excess of pectin (n+/n- ratio = 0.25). By contrast, the microparticles with an excess of pectin (n+/n- ratio 0.25) loaded with $FeSO_4$ showed a greater uptake by the cells. Even when we do not have currently an explanation to account for the observed differences, it is evident that the interplay between the interactions of ferric and ferrous and organic complexed iron with both chitosan and pectin in the complexed particles, along with the cellular uptake of the particle themselves, is what dictates the net cellular uptake of iron. Further mechanistic studies are necessary to fully uncover the phenomena at play.

Several studies have demonstrated that chitosan enhances the absorption of poorly permeable drugs when included in nano- and microparticle formulations for transmucosal delivery, an effect that could be attributed to its mucoadhesive properties (Fonte, Nogueira, Gehm, Ferreira, & Sarmento, 2011). Chitosan has positive charges due to the amine groups found in its structure. It permits a strong electrostatic interaction on the cell surface and consequently muco- and bio-adherence, leading to an increase on absorption

(Zariwala et al., 2013a). Other studies have demonstrated the enhancing effect of chitosan in their test systems. Zariwala et al. (2013a), studying solid lipid particles coated with chitosan, obtained higher absorption of iron than from chitosan-free systems. Yang et al. (2017) evaluated a system composed of ferritin glycosylated by chitosan to encapsulate catechin, a bioactive polyphenolic compound, and found an improvement on absorption when compared to the results with free catechin.

The iron uptake from OPN extract was also confirmed when comparing the results with those of $FeSO_4$ solution (containing equivalent doses of iron), in both cases free of particles. It was evidenced that OPN extract had lower uptake than $FeSO_4$. Meanwhile, it should be considered that the amount of total iron from OPN extract available to Caco-2 cells was 1522 ng mL^{-1} of which ~495 ng mL^{-1} is ferric (Fe^{2+}) iron. Hence the Caco-2 cells had absorbed ~35 % of the Fe^{2+} while for $FeSO_4$ solution, the absorption would be ~33 %. These results suggests that the proposed system could be a potentially promising alternative to iron supplementation in the future. A negative control using only serum-free MEM was used to demonstrate lack of iron uptake under these conditions.

The cells were treated with increasing concentrations of $FeSO_4$ solution (from 5.0 to 80.0 µM) with the purpose of evaluating a dose-response effect. The measured ferritin concentration increased from 53.08 to 785.31 ng mg^{-1} of cell protein and the data could be described by a function corresponding for one site specific binding with Hill slope (available from GraphPad), given as:

$$Y = B_{max} * X^h / \left({K_d}^h + X^h\right)$$

where Y = ferritin concentration (ng mg^{-1} of cell protein), B_{max} is the maximum specific binding (ng mg^{-1} of cell protein) extrapolated to high concentrations; K_d is the concentration of iron needed to achieve a half-maximum binding equilibrium, and h is the Hill slope. The best-fit values of the three parameters calculated were B_{max} = 776.4 ± 12.93, K_d = 13.12 ± 0.40, and h = 2.912 ± 0.21 (R^2 = 0.999) (Figure S1). The value of the Hill slope (h = 2.912) reflects a cooperative multiple binding sites process. The observed saturation-dose dependent process is fully consistent with the known mechanism of cellular iron uptake. For this to take place, ferrous iron first must be oxidized into the ferric form to bind transferrin. The Fe^{3+}-transferrin attaches to transferrin receptors (TfR2) on the cell membrane to be subsequently endocytosed.

Ferritin is the primary storage form of iron in cells. Hence, the concentration of ferritin reports on the overall uptake of iron. Our results are in agreement with other published work that indicated an ideal concentration for iron uptake to be 20 µM (Zariwala et al., 2013b).

4. CONCLUSION

In summary, we have gained proof-of-concept of the preparation and characterisation of iron-loaded chitosan-pectin microparticles as a potential platform for iron delivery and food supplementation/fortification, using an aqueous extract from a non-conventional edible Brazilian plant, OPN (Pereskia aculeata Miller). Microparticle characterisation evidenced the charge ratio (n+/n–) 5.00 and chitosan H-DA (22.3 %) as the optimal formulation. The average hydrodynamic diameter of the particles spanned < ~1000 to 2500 nm, production yield (~ 26 %), iron association efficiency (~60 %), spherical shape and stability in DMEM, SGF and SIF. Findings from cytotoxicity experiments revealed that all formulations involving particles at different iron concentration presented cell viability above ~80 %. The results showed Caco-2 cell iron uptake from microparticles at levels slightly close to those particles prepared using $FeSO_4$. An excess of chitosan in the proposed system evidenced the influence of this polymer to improve the iron uptake. This study should provide important insights related to the use of iron bioavailable from plants, confirmed by the good absorption properties in vitro. Iron-loaded chitosan-pectin microparticles offer a potential and versatile system that could provide an attractive drug delivery process for traditional oral iron treatment.

5. ACKNOWLEDGMENTS

This study was performed with the financial support of the São Paulo Research Foundation (FAPESP) scholarships (grant#2016/18142-4 and grant#2015/07872-9) and Coordination for the Improvement of Higher Education Personnel (CAPES). The authors would like to thank the kind collaboration of Dr. Yadira Gonzalez with the determination of chitosan molecular weight and Dr. Jose Villa for the support during the cell culture studies.

6. REFERENCES

Al Hassan, N. N. (2015). The prevalence of iron deficiency anemia in a Saudi University female students. Journal of Microscopy and Ultrastructure, 3(1), 25-28.

https://doi.org/10.1016/j.jmau.2014.11.003.
Alencastre, J. B., Bentley, M. V. L. B., Garcia, F. S., De Moragas, M., Viladot, J. L., & Marchetti, J. M. (2006). A study of the characteristics and in vitro permeation properties of CMC/chitosan microparticles as a skin delivery system for vitamin E. Brazilian Journal of Pharmaceutical Sciences, 42(1), 69-76. http://dx.doi.org/10.1590/S1516-93322006000100007.
Almeida, M. E. F., & Corrêa, A. D. (2012). Utilização de cactáceas do gênero Pereskia na alimentação humana em um município de Minas Gerais. Ciência Rural, 42(4), 751-756. http://dx.doi.org/10.1590/S0103-84782012000400029.
Andreani, T., Miziara, L., Lorenzon, E. N., Souza, A. L. R., Kiill, C. P., Fangueiro, J. F., Garcia, M. L., Gremiao, P. D., Silva, A. M., & Souto, E. B. (2015). Effect of mucoadhesive polymers on the in vitro performance of insulin-loaded silica nanoparticles: Interactions with mucin and biomembrane models. European Journal of Pharmaceutics and Biopharmaceutics, 93, 118-126. https://doi.org/10.1016/j.ejpb.2015.03.027.
Andrews, N. C. (1999). Disorders of iron metabolism. The New English Journal of Medicine, 341, 1986-1995. https://doi.org/10.1056/NEJM199912233412607.
Argüelles-Monal, W., Cabrera, G., Peniche, C., & Rinaudo, M. (2000). Conductimetric study of the interpolyelectrolyte reaction between chitosan and polygalacturonic acid. Polymer, 41(7), 2373-2378. https://doi.org/10.1016/S0032-3861(99)00396-1.
Bagre, A. O., Jain, K., & Jain, N. K. (2013). Alginate coated chitosan core shell nanoparticles for oral delivery of enoxaparin: In vitro and in vivo assessment. International Journal of Pharmaceutics, 456(1), 31-40. https://doi.org/10.1016/j.ijpharm.2013.08.037.
Bhatia, S. C., & Ravi, N. A. (2003). Mössbauer study of the interaction of chitosan and D-glucosamine with iron and its relevance to other metalloenzymes. Biomacromolecules, 4(3), 723-727. https://doi.org/10.1021/bm020131n.
Bayat, A., Dorkoosh, F. A., Dehpour, A. R., Moezi, L., Larijani, B., Junginger, H. E., & Rafiee-Tehrani, M. (2008). Nanoparticles of quaternized chitosan derivatives as a carrier for colon delivery of insulin: Ex vivo and in vivo studies. International Journal of Pharmaceutics, 356(1-2), 259-266. https://doi.org/10.1016/j.ijpharm.2007.12.037.
Belscak-Cvitanovic, A., Đordevic, V., Karlovic, S., Pavlovic, V., Komes, D., Jezek, D., Bugarski, B., & Nedovic, V. (2015). Protein-reinforced and chitosan-pectin coated alginate microparticles for delivery of flavan-3-ol antioxidants and caffeine from green

764 tea extract. Food Hydrocolloids, 51, 361-374.
765 https://doi.org/10.1016/j.foodhyd.2015.05.039.
766 Bernabé, P., Peniche, C., & Argüelles-Monal, W. (2005). Swelling behavior of
767 chitosan/pectin polyelectrolyte complex membranes. Effect of thermal cross-linking.
768 Polymer Bulletin, 55(5), 367-375. https://doi.org/10.1007/s00289-005-0439-5.
769 Berger, J., Reist, M., Mayer, J. M., Felt, O., & Gurny, R. (2004). Structure and
770 interactions in chitosan hydrogels formed by complexation or aggregation for biomedical
771 applications. European Journal of Pharmaceutics and Biopharmaceutics, 57(1), 35-52.
772 https://doi.org/10.1016/S0939-6411(03)00160-7.
773 Calvo, P., Remunan-Lopez, C., Vila-Jato, J. L., & Alonso, M. J. (1997). Novel
774 hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. Journal of
775 Applied Polymer Science, 63(1), 125-132. https://doi.org/10.1002/(SICI)1097-
776 4628(19970103)63:1<125::AID-APP13>3.0.CO;2-4.
777 Carlan, I. C., Estevinho, B. N., & Rocha, F. (2017). Study of microencapsulation and
778 controlled release of modified chitosan microparticles containing vitamin B12. Powder
779 Technology, 318, 162-169. https://doi.org/10.1016/j.powtec.2017.05.041.
780 Cerchiara, T., Abruzzo, A., Di Cagno, M., Bigucci, F., Bauer-Brandl, A., Parolin, C.,
781 Vitali, B., Gallucci, M. C., & Luppi, B. (2015). Chitosan based micro- and nanoparticles
782 for colon-targeted delivery of vancomycin prepared by alternative processing methods.
783 European Journal of Pharmaceutics and Biopharmaceutics, 92, 112-119.
784 https://doi.org/10.1016/j.ejpb.2015.03.004.
785 Chen, M-C., Wong, H-S., Lin, K-J., Chen, H-L., Wey, S-P., Sonaje, K., Lin, Y-H., Chu,
786 C-Y., & Sung, H-W. (2009). The characteristics, biodistribution and bioavailability of a
787 chitosan-based nanoparticulate system for the oral delivery of heparin. Biomaterials,
788 30(34), 6629-6637. https://doi.org/10.1016/j.biomaterials.2009.08.030.
789 Chen, P-H., Kuo, T-Y., Kuo, J-Y., Tseng, Y-Po., Wang, D-M., Lai, J-Y., & Hsieh, H-J.
790 (2010). Novel chitosan–pectin composite membranes with enhanced strength,
791 hydrophilicity and controllable disintegration. Carbohydrate Polymers, 82, 1236-1242.
792 https://doi.org/10.1016/j.carbpol.2010.06.057.
793 Costalat, M., David, L., & Delair, T. (2014). Reversible controlled assembly of chitosan
794 and dextran sulfate: A new method for nanoparticle elaboration. Carbohydrate Polymers,
795 102 (1), 717-726. https://doi.org/10.1016/j.carbpol.2013.10.098

796 Crichton, R. (2001). Inorganic biochemistry of iron metabolism: from molecular
797 mechanisms to clinical consequences. (2nd ed.). Chichester: John Wiley & Sons Ltd,
798 (Chapter 2).
799 Crichton, R. R., Wilmet, S., Legssyer, R., & Ward, R. J. (2002). Molecular and cellular
800 mechanisms of iron homeostasis and toxicity in mammalian cells. Journal of Inorganic
801 Biochemistry, 91, 9-18. http://dx.doi.org/10.1016/S0162-0134(02)00461-0.
802 Fonte, P., Nogueira, T., Gehm, C., Ferreira, D., & Sarmento, B. (2011). Chitosan-coated
803 solid lipid nanoparticles enhance the oral absorption of insulin. Drug Delivery and
804 Translational Research, 1(4), 299-308. https://doi.org/10.1007/s13346-011-0023-5.
805 Fuenzalida, J. P., Weikert, T., Hoffmann, S., Vila-Sanjurjo, C., Moerschbacher, B. M.,
806 Gropper S. S., Smith J. L, & Groff, J. K. (2009). Advanced Nutrition and Human
807 Metabolism. (5th ed.). Wadsworth: Belmont.
808 Ge, J., Yue, P., Chi, J., Liang, J., & Gao, X. (2018). Formation and stability of
809 anthocyanins-loaded nanocomplexes prepared with chitosan hydrochloride and
810 carboxymethyl chitosan. Food Hydrocolloids, 74, 23-31.
811 https://doi.org/10.1016/j.foodhyd.2017.07.029.
812 George, M., & Abraham, T. E. (2006). Polyionic hydrocolloids for the intestinal delivery
813 of protein drugs: alginate and chitosan: a review. Journal of Controlled Release, 114(1),
814 1-14. https://doi.org/10.1016/j.jconrel.2006.04.017.
815 Glahn, R. P., Gangloff, M. B., Van Campen, D. R., Miller, D. D., Wien, E. M., & Norvell,
816 W. A. (1995). Bathophenanthrolene disulfonic acid and sodium dithionite effectively
817 remove surface-bound iron from Caco-2 cell monolayers. The Journal of Nutrition,
818 125(7), 1833-1840. https://doi.org/10.1093/jn/125.7.1833.
819 Goycoolea, F. M., Brunel, F., Gueddari, N. E., Coggiola, A., Lollo, G., Moerschbacher,
820 B. M., Remuñán-López, C., Delair, T., Domard, A., & Alonso, M. J. (2016). Physical
821 properties and stability of soft gelled chitosan-based nanoparticles. Macromolecular
822 Bioscience, 16(12), 1873-1882. https://doi.org/10.1002/mabi.201600298.
823 Goycoolea, F. M., Valle-Gallego, A., Stefani, R., Menchicchi, B., David, L., Rochas, C.,
824 Santander-Ortega, M. J., & Alonso, M. J. (2012). Chitosan-based nanocapsules: Physical
825 characterization stability in biological media and capsaicin encapsulation. Colloid and
826 Polymer Science, 290(14), 1423-1434. https://doi.org/10.1007/s00396-012-2669-z.
827 Gronner, A., Silva, V. D., & Maluf, W. R. (1999). Ora-pro-*nobis (Pereskia aculeata): "a*
828 *carne de pobre".* Lavras: UFLA (Boletim Técnico de Hortaliças, 37).
829 Gursoy, N., Garrigue, J. S., Razafindratsita, A., Lambert, G., & Benita, S. (2003).

Excipient effects on in vitro cytotoxicity of a novel paclitaxel self-emulsifying drug delivery system. Journal of Pharmaceutical Science, 92(12), 2411-2418. https://doi.org/10.1002/jps.10501.

Hermida, L. G., Roig, A., Bregni, C., Sabés-Xamaní, M., & Barnadas-Rodríguez, R. (2010). Preparation and characterization of iron-containing liposomes: their effect on soluble iron uptake by Caco-2 cells. Journal of Liposome Research, 21(3), 203-212. https://doi.org/10.3109/08982104.2010.517536.

Hu, Q., Bae, M., Fleming, E., Lee, J-Y., & Luo, Y. (2019). Biocompatible polymeric nanoparticles with exceptional gastrointestinal stability as oral delivery vehicles for lipophilic bioactives. Food Hydrocolloids, 89, 386-395. https://doi.org/10.1016/j.foodhyd.2018.10.057.

Huang, X., Liu, Y., Zou, Y., Liang, X., Peng, Y., McClements, D. J., & Hu, K. (2019). Encapsulation of resveratrol in zein/pectin core-shell nanoparticles: Stability, bioaccessibility, and antioxidant capacity after simulated gastrointestinal digestion. Food Hydrocolloids, 93, 261-269. https://doi.org/10.1016/j.foodhyd.2019.02.

Hurrell, R. F., Lynch, S., Bothwell, T., Cori, H., Glahn, R., Hertrampf, E., Kratky, Z., Miller, D., Rodenstein, M., Streekstra, H., Teucher, B., Turner, E., Yeung, C. K., & Zimmermann, M. B. (2004). Enhancing the absorption of fortification iron. A sustain task force report. International Journal of Vitamin Nutrition Research, 74(6), 387-401. https://doi.org/10.1024/0300-9831.74.6.387.

Iannone, M., Mare, R., Paolino, D., Gagliardi, A., Froiio, F., Cosco, D., & Fresta, M. (2017). Characterization and in vitro anticancer properties of chitosan-microencapsulated flavan-3-ols-rich grape seed extracts. International Journal of Biological Macromolecules, 104(PtA), 1039-1045. https://doi.org/10.1016/j.ijbiomac.2017.07.022.

Kleine-Brüggeney, H., Zorzi, G. K., El-Gueddari, N. E., Moerschbacher, B. M., & Goycoolea, F. M. (2015). A rational approach towards the design of chitosan-based nanoparticles obtained by ionotropic gelation. Colloids Surface B, 135, 99-108. https://doi.org/10.1016/j.colsurfb.2015.07.016.

Kulikouskaya, V. I., Lazouskaya, M. E., & Agabekov, V. E. (2019). Features of the Formation of Interpolyelectrolyte Complexes Based on Chitosan and Pectin. Theoretical and Experimental Chemistry, 54(6), 375-385. https://doi.org/10.1007/s11237-019-09584-8.

862 Kumar, A., & Ahuja, M. (2013). Carboxymethyl gum kondagogu–chitosan
863 polyelectrolyte complex nanoparticles: preparation and characterization. International
864 Journal of Biological Macromolecules, 62, 80-84.
865 https://doi.org/10.1016/j.ijbiomac.2013.08.035.
866 Lavertu, M., Xia, Z., Serreqi, A. N., Berrada, M., Rodrigues, A., Wang, D., Buschmann,
867 M. D., & Gupta, A. (2003). A validated ^{1}H NMR method for the determination of the
868 degree of deacetylation of chitosan. Journal of Pharmaceutical and Biomedical Analysis,
869 32(6), 1149-1158. https://doi.org/10.1016/S0731-7085(03)00155-9.
870 Lima Junior, F. A., Conceição, M. C., Resende, J. V., Junqueira, L. A., Pereira, C. G., &
871 Prado, M. E. T. (2013). Response surface methodology for optimization of the mucilage
872 extraction process from Pereskia aculeata Miller. Food Hydrocolloids, 33(1), 38-47.
873 https://doi.org/10.1016/j.foodhyd.2013.02.012.
874 Lea, T. (2015). Caco-2 cell line. In K. Verhoeckx, P. Cotter, I. López-Expósito, C.
875 Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), The
876 impact of food bio-actives on gut health (pp. 103-111). Springer International Publishing.
877 Loretz, B., & Bernkop-Schnurch, A. (2007). In vitro cytotoxicty testing of non-thiolated
878 and thiolated chitosan nanoparticles for oral gene delivery. Nanotoxicology, 1(2), 139-
879 148. https://doi.org/10.1080/17435390701554200.
880 Luo, Y., Teng, Z., Li, Y., & Wang, Q. (2015). Solid lipid nanoparticles for oral drug
881 delivery: Chitosan coating improves stability, controlled delivery, mucoadhesion and
882 cellular uptake. Carbohydrate Polymers, 122, 221-229.
883 https://doi.org/10.1016/j.carbpol.2014.12.084.
884 Luo, Y., & Wang, Q. (2014). Recent development of chitosan-based polyelectrolyte
885 complexes with natural polysaccharides for drug delivery. International Journal of
886 Biological Macromolecules, 64, 353-367.
887 https://doi.org/10.1016/j.ijbiomac.2013.12.017.
888 Maciel, V. B. V., Yoshida, C. M. P., & Franco, T. T. (2015). Chitosan/pectin
889 polyelectrolyte complex as a pH indicator. Carbohydrate Polymers, 132, 537-545.
890 https://doi.org/10.1016/j.carbpol.2015.06.047.
891 Maciel, V. B. V., Yoshida, C. M. P., Pereira, S. M. S. S., Goycoolea, F. M., & Franco, T.
892 T. (2017). Electrostatic self-assembled chitosan-pectin nano- and microparticles for
893 insulin delivery. Molecules, 22(10), E1707. https://doi.org/10.3390/molecules22101707.

894 Maciel, V. B. V., Yoshida, C. M. P., & Goycoolea, F. M. (2018). Agronomic Cultivation,
895 Chemical Composition, Functional Activities and Applications of Pereskia Species - A
896 Mini Review, Current Medicinal Chemistry, in press, 2018.
897 https://doi.org/10.2174/0929867325666180926151615.
898 Mallick, S. P., Singh, B. N., Rastogi, A., & Srivastava, P. (2018). Design and evaluation
899 of chitosan/poly(L-lactide)/pectin based composite scaffolds for cartilage tissue
900 regeneration. International Journal of Biological Macromolecules, 112, 909-920.
901 https://doi.org/10.1016/j.ijbiomac.2018.02.049.
902 Martins, J. G., Camargo, S. E. A., Bishop, T. T., Popat, K. C., Kipper, M. J., & Martins,
903 A. F. (2018). Pectin-chitosan membrane scaffold imparts controlled stem cell adhesion
904 and proliferation. Carbohydrate Polymers, 197, 47-56.
905 https://doi.org/10.1016/j.carbpol.2018.05.062.
906 Martnez-Navarrete, N., Camacho, M. M., Martnez-Lahuerta, J., Martnez-Monzo, J., &
907 Fito, P. (2002). Iron deficiency and iron fortified foods – a review. Food Research
908 International, 35(2-3), 225-231. https://doi.org/10.1016/S0963-9969(01)00189-2.
909 Menchicchi, B., Fuenzalida, J. P., Bobbili, K. B., Hensel, A., Swamy, M. J., & Goycoolea,
910 F. M. (2014). Structure of chitosan determines its interactions with mucin.
911 Biomacromolecules, 15(10), 3550-3558. https://doi.org/10.1021/bm5007954.
912 Meng, X., Tian, F., Yang, J., He, C-N., Xing, N., & Li, F. (2010). Chitosan and alginate
913 polyelectrolyte complex membranes and their properties for wound dressing application.
914 Journal of Materials Science: Materials in Medicine, 21(5), 1751-1759.
915 https://doi.org/10.1007/s10856-010-3996-6.
916 Nguyen, S., Winnik, F. M., & Buschmann, M. D. (2009). Improved reproducibility in the
917 determination of the molecular weight of chitosan by analytical size exclusion
918 chromatography. Carbohydrate Polymers, 75(3), 528-533.
919 https://doi.org/10.1016/j.carbpol.2008.08.013.
920 Nishijima, T., Iwai, K., Saito, Y., Takida, Y., & Matsue, H. Chronic ingestion of apple
921 pectin can enhance the absorption of quercetin. Journal of Agricultural and Food
922 Chemistry, 57(6), 2583-2587. https://doi.org/10.1021/jf803547h.
923 Niu, Z., Loveday, S. M., Barbe, V., Thielen, I., He, Y., & Singh, H. (2019). Protection of
924 native lactoferrin under gastric conditions through complexation with pectin and chitosan.
925 Food Hydrocolloids, 93, 120-130.
926 https://doi.org/https://doi.org/10.1016/j.foodhyd.2019.02.020.
927 Okonogi, S., Duangrat, C., Anuchpreeda, S., Tachakittirungrod, S., &

928 Chowwanapoonpohn, S. (2007). Comparison of antioxidant capacities and cytotoxicities
929 of certain fruit peels. Food Chemistry, 103(3), 839-846.
930 https://doi.org/10.1016/j.foodchem.2006.09.034.
931 Oliveira, D. C. S., Wobeto, C., Zanuzo, M. R., & Severgnini, C. (2013). Composição
932 mineral e teor de ácido ascórbico nas folhas de quatro espécies olerícolas não-
933 convencionais. Horticultura Brasileira, 31(3), 472-475.
934 http://dx.doi.org/10.1590/S0102-05362013000300021.
935 Oliveira, P. M., Matos, B. N., Pereira, P. A. T., Gratieri, T., Faccioli, L. H., Cunha-Filho,
936 M. S. S., & Gelfuso, G. M. (2017). Microparticles prepared with 50–190 kDa chitosan as
937 promising non-toxic carriers for pulmonary delivery of isoniazid. Carbohydrate
938 Polymers, 174, 421-431. https://doi.org/10.1016/j.carbpol.2017.06.090.
939 Ribeiro, L. N. M., Alcântara, A. C. S., Darder, M., Aranda, P., Araújo-Moreira, F. M., &
940 Ruiz-Hitzky, E. (2014). Pectin-coated chitosan-LDH bionanocomposite beads as
941 potential systems for colon-targeted drug delivery. International Journal of
942 Pharmaceutics, 463(1), 1-9. https://doi.org/10.1016/j.ijpharm.2013.12.035.
943 Rutz, J. K., Borges, C. D., Zambiazi, R. C., da Rosa, C. G., & da Silva, M. M. (2016).
944 Elaboration of microparticles of carotenoids from natural and synthetic sources for
945 applications in food. Food Chemistry, 202, 324-333.
946 https://doi.org/10.1016/j.foodchem.2016.01.140.
947 Saha, L., Pandhi, P., Gopalan, S., Malhotra, S., & Saha, P. K. (2007). Comparison of
948 efficacy, tolerability, and cost of iron polymaltose complex with ferrous sulphate in the
949 treatment of iron deficiency anemia in pregnant women. Medscape General Medicine,
950 9(1), 1. PMID: 17435611.
951 Santander-Ortega, M. J., Peula- García, J. M., Goycoolea, F. M., & Ortega-Vinuesa, J. L.
952 (2011). Chitosan nanocapsules: effect of chitosan molecular weight and acetylation
953 degree on their electrokinetic and stability behaviour. Colloids Surfaces B: Biointerfaces
954 82, 571-580. https://doi.org/10.1016/j.colsurfb.2010.10.019
955 Santos-Carballal, B., Aaldering, L. K., Ritzefeld, M., Pereira, S., Sewald, N.,
956 Moerschbacher, B. M., Götte, M., & Goycoolea, F. M. (2015). Physicochemical and
957 biological characterization of chitosan-microRNA nanocomplexes for gene delivery to
958 MCF-7 breast cancer cells. Scientific Reports, 5, 13567.
959 https://doi.org/10.1038/srep13567.
960 Schümann, K., Ettle, T., Szegner, B., Elsenhans, B., & Solomons, N. W. (2007). On risks
961 and benefits of iron supplementation recommendations for iron intake revisited. Journal

962 of Trace Elements in Medicine and Biology, 21(3), 147-168.
963 https://doi.org/10.1016/j.jtemb.2007.06.002.
964 Sharma, A., Shree, B. G. S., Arora, S., & Kapila, S. (2017). Preparation of lactose-iron
965 complex and its cyto-toxicity, in-vitro digestion and bioaccessibility in Caco-2 cell model
966 system. Food Bioscience, 20, 125-130. https://doi.org/10.1016/j.fbio.2017.10.001.
967 Sharp, P. (2005). Methods and options for estimating iron and zinc bioavailability using
968 Caco-2 cell models: benefits and limitations. International Journal for Vitamin and
969 Nutrition Research, 75(6), 413-421. https://doi.org/10.1024/0300-9831.75.6.413.
970 Soleimani, N., & Abbaszadeh, N. (2011) Relationship between anaemia, caused from the
971 iron deficiency, and academic achievement among third grade high school female
972 students. International Conference on Education and Educational Psychology, 29, 1877-
973 1884. https://doi.org/10.1016/j.sbspro.2011.11.437.
974 Soliman, G. M., Zhang, Y. L., Merle, G., Cerruti, M., & Barralet, J. (2014). Hydrocaffeic
975 acid–chitosan nanoparticles with enhanced stability, mucoadhesion and permeation
976 properties. European Journal of Pharmaceutics and Biopharmaceutics, 88(3), 1026-
977 1037. https://doi.org/10.1016/j.ejpb.2014.09.003.
978 Somsook, E., Hinsin, D., Buakhrong, P., Teanchai, R., Mophan, N., Pohmakotr, M., &
979 Shiowatana, J. (2005). Interactions between iron(III) and sucrose, dextran, or starch in
980 complexes. Carbohydrate Polymers, 61, 281-287.
981 https://doi:10.1016/j.carbpol.2005.04.019
982 Standard Methods for the Examination of Water and Wastewater. (1999). American
983 Public Health Association (pp. 442-447).
984 Takeiti, C. Y., Antônio, G. C., Motta, E. M. P., Collares-Queiroz, F. P., & Park, K. J.
985 (2009). Nutritive evaluation of a non-conventional leafy vegetable (Pereskia aculeate
986 Miller). International Journal of Food Sciences and Nutrition, 60(Suppl. 1), 148-160.
987 https://doi.org/10.1080/09637480802534509.
988 Trapani, A., Di Gioia, S., Ditaranto, N., Cioffic, N., Goycoolea, F. M., Carbone, A.,
989 Garcia-Fuentes, M., Conese, M., & Alonso, M.J. (2013). Systemic heparin delivery by
990 the pulmonary route using chitosan and glycol chitosan nanoparticles. International
991 Journal of Pharmaceutics, 447(1-2), 115-123.
992 https://doi.org/10.1016/j.ijpharm.2013.02.035.
993 USP-NF. (2019). United States Pharmacopeia 42-National Formulary 37. Rockville:
994 United States Pharmacopeial Convention.
995 Xia, S., & Xu, S. (2005). Ferrous sulfate liposomes: preparation, stability and application

in fluid milk. Food Research International, 38(3), 289-296. https://doi.org/10.1016/j.foodres.2004.04.010.

Vaarum, K. M., & Smidsrod, O. (2005). Structure-property relationship in chitosans. In S. Dumitriu (Ed.), Polysaccharides: Structural Diversity and Functional Versatility (pp. 625-660), New York: Marcel Dekker Inc.

Yang, R., Liu, Y., Gao, Y., Wang, Y., Blanchard, C., & Zhou, Z. (2017). Ferritin glycosylated by chitosan as a novel EGCG nano-carrier: Structure, stability, and absorption analysis. International Journal of Biological Macromolecules, 105(Pt 1), 252-261. https://doi.org/10.1016/j.ijbiomac.2017.07.040.

Yuan, D., Jacquier, J. C., & O'Riordan, E. D. (2018). Entrapment of proteins and peptides in chitosan-polyphosphoric acid hydrogel beads: a new approach to achieve both high entrapment efficiency and controlled in vitro release. Food Chemistry, 239, 1200-1209. https://doi.org/10.1016/j.foodchem.2017.07.021.

Zhang, L., Wang, J., Ni, C., Zhang, Y., & Shi, G. (2016). Preparation of polyelectrolyte complex nanoparticles of chitosan and poly(2-acry1amido-2-methylpropanesulfonic acid) for doxorubicin release. Material Science and Engineering: C, 58, 724-729. https://doi.org/10.1016/j.msec.2015.09.044.

Zhang, H., & Zhao, Y. (2015). Preparation, characterization and evaluation of tea polyphenole-Zn complex loaded β-chitosan nanoparticles. Food Hydrocolloids, 48, 260-273. https://doi.org/10.1016/j.foodhyd.2015.02.015.

Zariwala, M. G., Elsaid, N., Jackson, T. L., López, F. C., Farnaud, S., Somavarapu, S., & Renshaw, D. (2013a). A novel approach to oral iron delivery using ferrous sulphate loaded solid lipid nanoparticles. International Journal of Pharmaceutics, 456(2), 400-407. https://doi.org/10.1016/j.ijpharm.2013.08.070.

Zariwala, M. G., Somavarapu, S., Farnaud, S., & Renshaw, D. (2013b). Comparison study of oral iron preparations using a human intestinal model. Scientia Pharmaceutica, 81(4), 1123-1139. https://doi.org/10.3797/scipharm.1304-03.

Zimmermann, M. B., & Hurrell, R. F. (2007). Nutritional iron deficiency. Lancet, 370(9586), 511-520. https://doi.org/10.1016/S0140-6736(07)61235-5.

Table 1. Mineral content present in ora-pro-nobis (OPN) extract.

Minerals		Content
Manganese	($\mu g\ L^{-1}$)	4593.00
Iron		2030.00
Copper		105.00
Zinc		373.00
Calcium	($mg\ L^{-1}$)	6.03
Magnesium		79.10
Potassium		465.00
Sulphur		26.70
Phosphorous		20.20

Table 2. Production yield of chitosan-pectin microparticles loaded with ora-pro-nobis (OPN) extract prepared at different charge ratios (n+/n−) and comprising chitosans of varying degree of acetylation.

Chitosan (DA)	Charge ratio (n+/n−)	Production yield (%)
L-DA (8.6 %)	0.25	20.41 ± 0.96^{b}
	5.00	25.01 ± 0.84^{a}
H-DA (22.3 %)	0.25	26.38 ± 0.63^{a}
	5.00	26.09 ± 0.41^{a}

Different letters differ statistically ($p < 0.05$) by Tukey's test. Results are mean with SE of three independent experiments. L-DA = low degree of acetylation. H-DA = high degree of acetylation.

Table 3. Iron association efficiency from different sources (ora-pro-nobis extract (OPN) and $FeSO_4$ solution) at the microparticles prepared in different charge ratios (n+/n−) and chitosan with degree of acetylation 22.3 %.

Samples	**Charge ratio (n+/n−)**	**Iron association efficiency (%)**
Chitosan/pectin/OPN extract	0.25	33.99 ± 2.54^{d}
Chitosan/pectin/OPN extract	5.00	59.56 ± 3.33^{b}
Chitosan/pectin/$FeSO_4$ solution	0.25	45.78 ± 2.87^{c}
Chitosan/pectin/$FeSO_4$ solution	5.00	63.21 ± 4.69^{a}

Results are mean with SE of three independent experiments. Different letters differ statistically ($p < 0.05$) by Tukey's test.

Table 4. Caco-2 cells iron uptake from microparticles, ora-pro-nobis (OPN) extract and $FeSO_4$ solution.

Samples	Charge ratio (n+/n–)	Ferritin (ng mg^{-1} of cell protein)
CS/PT/OPN extract	0.25	3.53 ± 0.21^{g}
CS/PT/OPN extract	5.00	6.81 ± 0.36^{f}
CS/PT/$FeSO_4$ solution	0.25	28.18 ± 1.97^{d}
CS/PT/$FeSO_4$ solution	5.00	16.41 ± 1.45^{e}
OPN extract[#1]	-	174.96 ± 15.44^{c}
$FeSO_4$ solution[#2]	-	508.79 ± 26.43^{a}
Negative control	-	0.89 ± 0.01^{h}

Different letters denote statistically significant differences ($p < 0.05$) by Tukey's test. Results are mean average ± SE of three independent experiments. [#1]Amount of total iron: 1522 ng mL^{-1}. [#2]Amount of iron Fe^{2+}: 1522 ng mL^{-1}. CS = chitosan, PT = pectin.

Table 5. Dose-response effect for Caco-2 cells iron uptake using $FeSO_4$ solution at different concentrations.

$FeSO_4$ solution (µM)	**Ferritin (ng mg^{-1} of cell protein)**
5.0	53.08 ± 3.85^{d}
10.0	233.58 ± 16.52^{c}
20.0	607.58 ± 28.54^{b}
50.0	744.41 ± 42.11^{a}
80.0	785.31 ± 39.05^{a}

Different letters differ statistically ($p < 0.05$) by Tukey's test. Results are mean with SE of three independent experiments.