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Lactoferrin-based nanoparticles as a vehicle for iron in food applications – Development and release profile

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ABSTRACT

This study aims at developing and characterizing bovine lactoferrin (bLf) nanoparticles as an iron carrier. bLf nanoparticles were characterized in terms of size, polydispersity index (PdI), electric charge (ζ-potential), morphology, structure and stability over time. Subsequently, iron release experiments were performed at different pH values (2.0 and 7.0) at 37 °C, in order to understand the release mechanism. bLf (0.2%, w/v) nanoparticles were successfully produced by thermal gelation (75 °C for 20 min). bLf nanoparticles with 35 mM $FeCl_3$ showed an iron binding efficiency value of approximately 20%. The nanoparticles were stable (i.e. no significant variation of size and PdI of the nanoparticles) for 76 days at 4 °C and showed to be stable between 4 and 60 °C and pH 2 and 11. Release experiments at pH 2 showed that iron release could be described by the linear superposition model (explained by Fick and relaxation phenomenon). On the contrary, the release mechanism at pH 7 cannot be described by either Fick or polymer relaxation behaviour. In general, results suggested that bLf nanoparticles could be used as an iron delivery system for future food applications.

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1. Introduction

Iron deficiency affects ca. two billion people worldwide in developing and mainly in developed countries (Martins et al., 2015). The best strategy to overcome this is to include in the diet a wide variety of foods rich in iron (Mason, Lotfi, Dalmiya, Spethuramen, & Deitchler, 2001). However, iron incorporated into complex food systems presents various problems such as oxidation and precipitation (Nicolai, Britten, & Schmitt, 2011; Van der Meer, Bovee-Oudenhoven, Sesink, & Kleibeuker, 1998; Wapnir, 1990). Thus, carrier systems that can actually transport and protect iron efficiently represent a field of great interest in food industry.

Several types of delivery systems at nanoscale have been developed in order to improve effectiveness and biocompatibility of bioactive compounds (Zariwala, Farnaud, Merchant, Somavarapu, & Renshaw, 2014), being nanoparticles one of these examples. These nanoparticles can be developed from natural (e.g. β-lactoglobulin and alginate) or synthetic (e.g. poly(*N*-isopropylacrylamide)) materials (Cerqueira et al., 2013; Fuciños et al., 2014). Additionally, they present a large surface area that can be used as a functionalization surface to specific targets, which are not accessible to macro- or microscaled particles (Cerqueira et al., 2014; Fuciños et al., 2014; Martins et al., 2015). In the food industry, the use of nanoparticles composed of proteins constitutes an interesting strategy for encapsulation and protection of micronutrients such as iron (Bourbon et al., 2015; Chen, Remondetto, & Subirade, 2006; Goldberg, Langer, & Xinqiao, 2007).

Gelling proteins, in particularly globular proteins (e.g. egg white, soy and whey proteins), have attracted much attention over the years due to their physico-chemical properties and industrial relevance (Clark, Kavanagh, & Ross-Murphy, 2001; Nicolai & Durand, 2013). Whey proteins (such as β-lactoglobulin and lactoferrin) have been widely used in food products due to their high nutritional value and gelation capacity (Ramos et al., 2014; Xiong & Kinsella, 1990). The bovine lactoferrin (bLf) from milk is a single-chain glycoprotein of the transferrin family with 703 amino acids, folded into two globular lobules, with a molecular weight of about 80 kDa and an isoelectric point (pI) around 8–9 (Levay & Viljoen, 1995). bLf is also of interest due to its biological properties such antibacterial, antiviral, immunomodulatory and high iron binding capacity (Adlerova, Bartoskova, & Faldyna, 2008; Brock, 2002; Levay & Viljoen, 1995). In order to form gels, bLf requires thermal treatment or addition of an agent for protein denaturation. The temperature, pH and ionic strength, for example, affect gel characteristics (Bourbon et al., 2015; Lefèvre & Subirade, 2000; Ziegler & Foegeding, 1990). Thermal gelation of proteins usually requires a heating step to unfold the native protein, followed by an aggregation process to give a three-dimensional

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network (at nano-scale). Gelation of proteins is one of the most used methods for development of protein aggregates and at high concentrations is used to form gels. When low concentrations are used is possible to produce nanoparticles (Bourbon et al., 2015; Ramos et al., 2014). After the heating step where protein denatures and polymerizes, the cooling step and subsequent salt addition are the following events, which induce protein aggregation (Remondetto, Paquin, & Subirade, 2002). Some examples of commonly used salts are calcium chloride ($CaCl_2$), chloride sodium (NaCl), magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$) and iron (III) chloride ($FeCl_3$) (Bourbon, Cerqueira, & Vicente, 2016; Roff & Foegeding, 1996). In this work, a ferric salt (i.e. $FeCl_3$) was chosen due to Fe^{3+} affinity to bLf which could address iron deficiency (Kanyshkova, Buneva, & Nevinsky, 2001). The gelation of proteins opens up interesting opportunities to produce innovative food-grade carriers for nutritional compounds (Remondetto et al., 2002). Therefore, bLf nanoparticles may be useful in food and pharmaceutical applications, e.g. to modify the optical or rheological properties of products, or to encapsulate and deliver bioactive ingredients. Moreover, understanding the molecules' release mechanisms by using mathematical modeling is essential for the design of nanoparticle-based delivery systems. This will allow foreseeing if the developed systems behaviour is appropriated to food products and consequently, to human consumption.

The main objectives of this study were the development and characterization of bLf-based nanoparticles as iron vehicle for food applications, and to highlight some of the factors that influence their properties. Additionally, iron release mechanisms from bLf nanoparticle at different pH were evaluated.

2. Material and methods

2.1. Materials

Purified bLf powder was obtained from DMV International (USA). This powder contained (expressed as a dry weigh percentage) 96% protein, 0.5% ash, 3.5% moisture and 0.012% iron (data supplied by the manufacturer). Iron chloride (III) ($FeCl_3$) (97% purity) was obtained from Panreac (Barcelona, Spain). Phosphate buffer saline (PBS) and hydrochloric acid (HCl) (36.5–38.0% purity) were purchased from Sigma–Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Potassium chloride and nitric acid (35% purity) were obtained from Merck (Darmstadt, Germany); sodium hydroxide (NaOH) was obtained from Fisher Scientific (UK). All samples were prepared with deionized water.

2.2. bLf nanoparticles preparation

2.2.1. Protein solution preparation

The nanoparticle preparation was based in the methodology used by other authors with some modifications (Bengoechea, Peinado, & McClements, 2011). Briefly, 0.2% (w/v) of bLf solution was dissolved in distilled water under agitation for 1 h at 25 °C. Then, pH of the solutions was adjusted to 7 using 1 M NaOH and/or 1 M HCl when necessary. According to Bengoechea et al. (2011), protein solutions (0.2% bLf, pH 7) under specific thermal conditions (i.e. 75 °C and holding time of 20 min) are favourable to the formation of nanoparticles. In order to study if the same conditions (i.e. protein concentration, pH, temperature and holding time) were adjusted to our work, these experimental conditions were used for the formation of bLf nanoparticles with $FeCl_3$ salt.

2.2.2. pH treatment

After agitation for 1 h, bLf solution was adjusted to different values of pH (4, 7 and 10) with 0.1 M HCl or NaOH (Riedel-de Haen, Germany). A holding time of 20 min at 75 °C was chosen based on preliminary work (results not shown) and in the optimum values reported (Bengoechea et al., 2011).

2.2.3. Thermal treatment

In order to study the effect of different temperatures in bLf behaviour, bLf protein solutions (0.2% bLf, pH 7) were subjected to different heat treatments: temperature (60–90 °C) and holding times (0–60 min).

2.2.4. Salt concentration

The effect of $FeCl_3$ salt on the protein aggregation in bLf solutions (0.2% bLf, pH 7) was evaluated by changing salt concentration of the solutions between 0 and 55 mM. Solutions were heated at 75 °C for 20 min and then different $FeCl_3$ concentrations were added.

2.3. bLf nanohydrogel characterization

2.3.1. Determination of size, polydispersity index (PdI) and ζ-potential

Size, PdI and ζ-potential of nanoparticles were determined using a dynamic light scattering (DLS) device (Zetasizer Nano ZS, Malvern Instruments, UK). The intensity-weighted size mean distribution (i.e. *Z*-average diameter) is reported for all size DLS data of the nanoparticles. The measurements were carried out at 25 °C. Each sample was analyzed in a folded capillary cell. Three true replicates were conducted, with three readings for each sample. Results are given as the average ± standard deviation of the experimental values.

2.3.2. Determination of protein solutions turbidity

The turbidity of protein solutions was analyzed using an UV/visible spectrophotometer at 600 nm (Synergy HT, Bio-Tek, Winooski, USA), and deionized water was used as blank sample.

The turbidity was analyzed in samples of protein solution (0.2% bLf, pH 7) that were heated at 60, 70, 75, 80 or 90 °C. From each solution, a sample was removed every 5 min, until the end of heating process (60 min). The measurements were made in triplicate and experimental values are given as the average ± standard deviation.

The turbidity of protein samples (0.2% bLf, pH 7, heating 75 °C for 20 min) with different $FeCl_3$ concentrations (0, 10, 35 and 55 mM) were analyzed. The experiments were performed in triplicate and the results are expressed in average ± standard deviation.

2.3.3. Transmission electron microscopy (TEM) measurement

The morphology of bLf nanoparticles with or without iron was evaluated on a Zeiss EM 902 A (Germany) microscope at a voltage of 80 kV. The samples were placed in carbon coated copper grids and left to dry at room temperature.

2.3.4. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of bLf powder, bLf powder and $FeCl_3$ mixture, bLf nanoparticles and bLf nanoparticles with $FeCl_3$ samples were determined using a FTIR spectrophotometer (Perkin-Elmer 16 PC spectrometer, Boston, USA). The samples were ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FTIR measurement. Spectral scanning was taken in the wavelength region between 4000 and 400 cm^{-1} and 16 scans were conducted. Each spectrum was baseline corrected and the transmittance was normalised.

2.3.5. Iron binding efficiency, loading capacity and yield efficiency

The iron binding efficiency and loading capacity to bLf nanoparticles was determined using the method described by other authors (Azevedo, Bourbon, Vicente, & Cerqueira, 2014). Briefly, bLf nanoparticles with iron were separated from free iron by molecular weight using Amicon® Ultra 0.5 mL 10 K filters (Millipore, Billerica, USA). 500 μL of sample were placed on the Amicon® filter and centrifuged at 14,000 rpm for 20 min (allowing free iron to pass through the filter). At last, the filter was centrifuged in an inverted position at 10,000 rpm for 5 min to allow collecting iron-binding bLf nanoparticles. The iron concentration was determined using Atomic Absorption Spectroscopy

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