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Encapsulation and controlled release of bioactive compounds in lactoferrin-glycomacropeptide nanohydrogels: Curcumin and caffeine as model compounds

Ana I. Bourbon*, Miguel A. Cerqueira, António A. Vicente

CEB – Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

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ABSTRACT

Curcumin and caffeine (used as lipophilic and hydrophilic model compounds, respectively) were successfully encapsulated in lactoferrin-glycomacropeptide (Lf-GMP) nanohydrogels by thermal gelation showing high encapsulation efficiencies (>90%). FTIR spectroscopy confirmed the encapsulation of bioactive compounds in Lf-GMP nanohydrogels and revealed that according to the encapsulated compound different interactions occur with the nanohydrogel matrix. The successful encapsulation of bioactive compounds in Lf-GMP nanohydrogels was also confirmed by fluorescence measurements and confocal laser scanning microscopy. TEM images showed that loaded nanohydrogels maintain their spherical shape with sizes of 112 and 126 nm for curcumin and caffeine encapsulated in Lf-GMP nanohydrogels, respectively; in both cases a polydispersity of 0.2 was obtained.

The release mechanisms of bioactive compounds through Lf-GMP nanohydrogels were evaluated at pH 2 and pH 7, by fitting the Linear Superimposition Model to the experimental data. The bioactive compounds release was found to be pH-dependent: at pH 2, relaxation is the governing phenomenon for curcumin and caffeine compounds and at pH 7 Fick's diffusion is the main mechanism of caffeine release while curcumin was not released through Lf-GMP nanohydrogels.

1. Introduction

The demand for encapsulation systems continues to grow as the food industry needs to preserve the benefits of active compounds and deliver them at specific conditions. Encapsulation of bioactive compounds in food industry can be used to: i) preserve functional properties, ii) improve the stability of compounds with low solubility in relevant (mostly aqueous) media, iii) mask undesirable flavours, iv) enhance health benefits of food products (i.e. development of functional foods), v) control the release of bioactive compounds at desired time and specific target, and vi) increase the bioavailability of bioactive compounds (Davidov-Pardo et al., 2015; Gunasekaran et al., 2007; Huang et al., 2010; Kayitmazer et al., 2013; Livney, 2010). Despite the interesting and unique properties that encapsulation can bring to food industry, its use is still a challenge mainly due to the need of using GRAS (generally recognized as safe) materials for the development of encapsulation systems.

Milk proteins are considered a vital macronutrient in food, offering the possibility of developing delivery systems for both hydrophilic and lipophilic bioactive compounds (Augustin and Oliver, 2014; Chen et al., 2006). Their biocompatibility, biodegradability, non-toxicity and ability to form hydrogels make them a relevant class of biopolymers to be used as vehicle of bioactive compounds (Fox, 2001), being one of the most promising systems used in food industry. Protein hydrogels are hydrophilic networks of swollen cross-linked polymers (Vermonden et al., 2012). The development of protein hydrogels at nano-scale is increasingly being studied for their attractive properties (e.g. ability to encapsulate different bioactive compounds, large surface area, response to environmental changes) in delivery systems (Oh et al., 2009; Yallapu et al., 2011). Lactoferrin (Lf) is an iron-binding glycoprotein with a isoelectric point around 8, found in various biological fluids of mammals. Lf is considered a multifunctional protein, playing several biological roles: antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory (Bokkhim et al., 2013; Embleton et al., 2013; González-Chávez et al., 2009; Madureira et al., 2007).

* Corresponding author.
E-mail address: isabelbourbon@gmail.com (A.I. Bourbon).

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This protein is also marketed as a nutritional supplement with high potential for biopharmaceutical applications (Balcão et al., 2013). Glycomacropeptide (GMP) is an acid glycosylated peptide that occurs naturally in bovine milk within the whey fraction. This peptide is considered an abundant protein, comprising around 20% of the total protein in sweet cheese whey (Neelima et al., 2013; Gustavo Hermes et al., 2013; Thomä-Worringer et al., 2006; van Calcar and Ney, 2012). GMP is sold as a food ingredient and has an excellent safety record based on widespread supplementation of foods infant formulas, both using whey proteins (Brück et al., 2006). Furthermore, GMP has functional properties such as: emulsification and foaming ability and can act in the inhibition of cholera toxin binding, anti-cariogenic and preventing intestinal infections (Neelima et al., 2013; Gustavo Hermes et al., 2013). Protein nanohydrogels, produced by interaction between Lf and GMP, were developed and characterized in a previous work (Bourbon et al., 2015). Due to their small size (170 nm) and high stability at various values of temperature and pH, these systems promised to be an excellent vehicle for encapsulation of bioactive compounds.

Curcumin (diferuloyl methane), a yellow lipid-soluble polyphenol is present in the rhizome of turmeric (*Curcuma longa* L.) and is widely used as a colouring agent in food. A wide range of biological attributes of curcumin such as antioxidative, anti-inflammatory, antiangiogenic, antiamyloid anticancer, antimicrobial, wound-healing and hepatoprotective properties have been well reported (Bhawana et al., 2011). However curcumin's poor solubility, stability, and bioavailability in aqueous media limits its efficient use as a bioactive compound. Efforts are being done to increase the bioavailability of this bioactive compound in aqueous solution, e.g. through its encapsulation in various delivery systems such as nanoemulsions (Sari et al., 2015), nanocapsules (Kittitheeranun et al.) and nanoparticles (Li et al., 2013).

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione), a white and water-soluble compound found in many plant species such as coffee and green tea, has received increasing attention in food and pharmaceutical industries due to its pharmacological properties, which comprise stimulation of the central nervous system, peripheral vasoconstriction, relaxation of the smooth muscle and myocardial stimulation (McLellan, 2006). Caffeine has frequently been used as a model compound, thus many formulations containing caffeine have been studied (Liédana et al., 2012; McLellan, 2006).

The present work aims at evaluating the ability of a protein-based nanohydrogel to encapsulate bioactive compounds with different water solubilities (curcumin as lipophilic compound and caffeine as hydrophilic compound) and evaluate their release mechanism through this matrix at different pH conditions, in an effort to reproduce at least partially the environment to be found during digestion, in view of future food applications.

2. Materials and methods

2.1. Materials

Lactoferrin-Lf was purchased from DMV International (USA) and Glycomacropeptide-GMP was kindly offered by Davisco Food International, INC. (Le Sueur, USA). To prepare the samples, it was used deionized water purified to a resistance of 15 MΩ, Millipore Corp. (France).

Hydrochloric acid was purchased from Panreac, Spain and sodium hydroxide was obtained from Riedel-de Haen (Germany). Hydrophilic model compound, caffeine was purchased from VWR (USA) and Amicon® Ultra-0.5 centrifugal filter of 3 kDa and 8 kDa devices from, Millipore Corp. (Ireland) were used.

Lipophilic model compound, curcumin was purchased from Sigma–Aldrich, St. Louis and pure ethanol was purchased from Panreac (Barcelona, Spain). Fluorescein isothiocyanate (FITC) was purchased from Fluka (Germany). Standard marker proteins from PageRuler™ Broad Range Unstained Protein Ladder, Lot ##002252 was purchased from Thermo Scientific (Lithuania).

2.2. Encapsulation of bioactive compounds in nanohydrogels

Nanohydrogels were prepared as described by Bourbon et al. (2015). Briefly, 2.5 μM of Lf and 8.33 μM of GMP were dissolved separately, in deionized water at 25 °C. The pH values of biopolymer solutions were separately adjusted to 5.0, with 0.1 mol L^{-1} of hydrochloric acid. Lf aqueous solution was added dropwise into GMP aqueous solution with gentle stirring until a final molar ratio (WR) of 1:7 (Lf:GMP) was reached.

2.2.1. Curcumin – lipophilic compound

Curcumin was used as lipophilic compound and a range of concentrations between of 0.005–0.18 mg mL^{-1} previously dissolved in absolute ethanol was added to the Lf-GMP mixture. After gentle stirring for 30 min, the mixture of Lf-GMP with curcumin was subsequently heated at 80 °C for 20 min in a water bath (closed system) to obtain a homogeneously dispersed nanohydrogel.

The unbound curcumin was removed by centrifuging the sample at 12 000 *g* for 20 min, which pulls down only the undissolved curcumin. The pellet of curcumin was carefully dissolved in ethanol and curcumin was quantified, spectrophotometrically, at 425 nm (Li et al., 2013). The amount of curcumin loaded in nanohydrogels was calculated by deducting the amount recovered in the ethanol fraction from the total amount of curcumin used. These results were used to calculate the EE (Equation (1)).

2.2.2. Caffeine – hydrophilic model compound

As in the case of curcumin, an amount of caffeine (hydrophilic model compound) ranging from 0.02 to 3 mg mL^{-1} previously dissolved in deionized water was gentle added to the Lf-GMP mixture. After gentle stirring for 30 min, the mixture of Lf-GMP with caffeine was subsequently heated at 80 °C for 20 min in a water bath (closed system) to obtain a homogeneously dispersed nanohydrogel.

The unbound caffeine was determined after separating the nanohydrogels with encapsulated caffeine from the solution with free caffeine. The separation was performed using an Amicon® Ultra-0.5 centrifugal filter 3 kDa device (Millipore Corp., Ireland). Briefly, 0.5 mL of sample was added to the Amicon® and centrifuged at 14 000 *g* during 10 min. After centrifugation a filtrate with free caffeine and a concentrate with nanohydrogels with encapsulated caffeine were obtained. The free caffeine was evaluated spectrophotometrically at 272 nm, which corresponds to the maximum absorbance peak of caffeine (Bagheri et al., 2014b), and the amount of free caffeine was calculated using an appropriate calibration curve: $y = 6.37x + 0.09$ ($R^2 = 0.98$) being y the Absorbance and x the concentration of free caffeine (mg.mL^{-1}). The obtained values were used to calculate the encapsulation efficiency (EE) (Equation (1)).