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Influence of chitosan coating on protein-based nanohydrogels properties and *in vitro* gastric digestibility

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ABSTRACT

Chitosan coating was applied in Lactoferrin (Lf)-Glycomacropeptide (GMP) nanohydrogels by layer-by-layer coating process. A volume ratio of 10 of Lf-GMP nanohydrogels (0.2 mg mL^{-1}, at pH 5.0) to chitosan (1 mg mL^{-1}, at pH 3) demonstrated to be the optimal condition to obtain stable nanohydrogels with size of 230 ± 12 nm, a PdI of 0.22 ± 0.02 and a ζ-potential of 30.0 ± 0.15 mV. Transmission electron microscopy (TEM) images showed that the application of chitosan coating in Lf-GMP did not affect the spherical shape of nanohydrogels and confirmed the low aggregation of nanohydrogels in solution. The analysis of chemical interactions between chitosan and Lf-GMP nanohydrogels were performed by Fourier transform infrared spectroscopy (FTIR) and by circular dichroism (CD) that revealed that a specific chemical interaction occurring between functional groups of protein-based nanohydrogels and active groups of the chitosan was established. The effect of chitosan coating on release mechanisms of Lf-GMP nanohydrogels at acid conditions (pH 2, 37 °C) was evaluated by the encapsulation of a model compound (caffeine) in these systems. Linear Superposition Model was used to fit the experimental data and revealed that Fick and relaxation mechanisms are involved in caffeine release. It was also observed that the Fick contribution increase with the application of chitosan coating. *In vitro* gastric digestion was performed with Lf-GMP nanohydrogels and Lf-GMP nanohydrogels with chitosan coating and it was observed that the presence of chitosan improve the stability of Lf and GMP (proteins were hydrolysed at a slower rate and were present in solution by longer time). Native electrophoreses revealed that the nanohydrogels without coating remained intact in solution until 15 min and with chitosan coating remained intact until 60 min, during gastric digestion.

1. Introduction

Protection of active compounds and the ability to maintain them active until release near the target cell tissue, is a great challenge for the food and pharmaceutical industries. Nanostructures are considered promising systems due their small dimensions that enables versatile advantages for targeted, site-specific delivery purposes as long as they can penetrate circulating systems and reach specific sites in the body at a suitable time (Cerqueira et al., 2014; Martins et al., 2015).

Protein nanohydrogels are considered an attractive vehicle to encapsulate and delivery different bioactive compounds, due their large network, low toxicity, high biodegradability, biocompatibility and ability to deliver bioactive compounds into and/or across the Gastro Intestinal (GI) mucosa (Somchue, Sermsri, Shiowatana, & Siripinyanond, 2009). Depending on the nature of the bioactive compounds incorporated in nanohydrogels it is possible to observe different release mechanisms during the digestion process. Hydrophilic compounds can be released from a protein matrix by diffusion, whereas lipophilic compounds are released mainly by enzymatic degradation of the protein matrix in the GI tract (Wang, Tian, & Chen, 2011). The degradation of protein matrix in GI tract is an obstacle to the delivery of the encapsulated compound at specific target (e.g. mouth, stomach, small intestine or colon). Nanostructures composed by proteins or peptides demonstrated to have a high level of GI degradation by digestive enzymes (Donato-Capel et al., 2014; Shaji & Patole, 2008).

Gastric conditions are determinant in digestion of protein structures. The stomach ensures the denaturation of proteins by the gastric acidity and also the proteins hydrolyse by pepsin (Yvon, Beucher, Scanff, Thirouin, & Pelissier, 1992). Nabil, Gauthier, Drouin, Poubelle, and Pouliot (2011) observed that almost of

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bovine whey protein extract (BWPE) was hydrolysed in gastric compartment and no intact whey protein was detected in intestinal compartments (Nabil et al., 2011). Moreover, these authors observed that after 1 h of gastric digestion 61% of BWPE was already hydrolysed. The degradation of proteins compromises the delivery of these active compounds in intestine were occurs the absorption. One of the strategies used to improve the stability of nanostructures in gastro conditions, is the application of a coating to prevent the hydrolysis of proteins by proteolytic enzymes (Somchue et al., 2009). Layer-by-Layer (LbL) deposition technique is one of the methods used in different templates (from hard and planar to rigid particles, and more recently in soft and porous templates such as nanohydrogels) to improve the stability, functional and mechanical properties of different structures (Boddohi, Almodóvar, Zhang, Johnson, & Kipper, 2010; Hirsjärvi, Qiao, Royere, Bibette, & Benoit, 2010; Kittitheeranun et al.; Kotov, 2003; Sato, Yoshida, Takahashi, & Anzai, 2011; Wong, Müller, Diez-Pascual, & Richtering, 2009). LbL assembly is based on the electrostatic interaction between oppositely charged polyelectrolytes alternatively adsorbed onto an appropriate template (Decher, 2003).

Chitosan, is a cationic polysaccharide obtained by deacetylation of chitin, which is the major constituent of exoskeleton of crustaceous animals. Chitosan is nontoxic, biodegradable, and biocompatible (Khopade & Caruso, 2004; Li, Wang & Wu, 1998). This polysaccharide is used to enhance bioactive compounds absorption in epithelium and its ability to protects structures in gastric environmental has been reported (Chew, Tan, Long, & Nyam, 2015; Rastall, 2010). In order to control the degradability of protein nanohydrogels and increase the residence time of proteins in the gastric conditions, chitosan coating has been applied in nanohydrogels composed by lactoferrin (Lf) and glycomacropeptide (GMP).

This study was carried out to evaluate the ability of chitosan coating to affect Lf-GMP nanohydrogels properties and their stability during gastric digestion. The influence of chitosan coating on Lf-GMP nanohydrogels was also evaluated in controlled release properties of caffeine. This study shows a successful attempt to use protein-based systems in combination with chitosan to allow protection and delivery of bioactive compounds to specific targets (e.g. intestinal epithelium) for bioavailability improvement.

2. Materials and methods

2.1. Materials

Purified Lf powder was obtained from DMV International (USA) and it is composed by 96% protein, 0.5% ash, 3.5% moisture and the an iron content is around 120 ppm (composition expressed as a dry weigh percentage). Commercial GMP was obtained from Davisco Food International, INC. (Le Sueur, USA) and its reported composition is: 82.5% protein, 1% fat, 7% ash and 7% moisture. Chitosan of low molecular weight (molecular weight ranging between 50 and 100 KDa and with a deacetylation degree ≥95%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Lactic acid (90%) was obtained from Acros Organics (Geel, Belgium). Caffeine was purchased from AnalaR NORMAPUR® (Ireland) and Amicon® Ultra-0.5 with a molecular cut-off 3 kDa centrifugal filter devices were purchased from Millipore Corp., Ireland. All the samples were dissolved in deionized water purified to a resistance of 15 MΩ (Millipore, France).

Cellu-Sep H1, dialysis membrane was obtained by Membrane filtration products, USA. To adjust the solutions pH it was used hydrochloric acid, purchased from Panreac, Spain.

For the simulated gastric juice, it was used pepsin from porcine gastric mucosa (600 U mL^{-1}), lipase from porcine pancreas (40 U mL^{-1}) and different salts (NaCl, KCl, $CaCl_2$ and $NaHCO_3$) to prepare the gastric electrolyte solutions; all of them purchased from Sigma (St. Louis, USA). All other chemicals used in this study were reagent grade.

2.2. Preparation of Lactoferrin-Glycomacropeptide nanohydrogels

Lf-GMP nanohydrogels were prepared as described in Bourbon et al. (2015). Briefly, 1.25 μM of Lf and 8.3 μM of GMP were dissolved separately, in deionized water purified 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 gently stirring until final molar ratio (MR) 1:7 of Lf to GMP. The Lf-GMP mixture solution was heated in a closed bath, at 80 °C during 20 min.

2.3. Preparation of chitosan coating on Lf-GMP nanohydrogels

The chitosan was assembled on the Lf-GMP nanohydrogels by LbL deposition technique.

After Lf-GMP nanohydrogels production process, the nanohydrogels were added to a chitosan solution (1 mg mL^{-1}, pH 3, dissolved in 1% of lactic acid) at different volume ratios (VR) of Lf-GMP nanohydrogels to chitosan, with constant stirring of 200 rpm during 15 min, creating the nanohydrogels with a coating.

2.4. Characterization of Lf-GMP nanohydrogels coated with chitosan

2.4.1. ζ-potential measurements

The ζ-potential of coated Lf-GMP nanohydrogels was determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK). Each sample was analysed in a folded capillary cell. The ζ-potential values are the average of nine successive measurements.

2.4.2. Size

Nanohydrogels with chitosan coating were characterized in terms of size distribution (by number) and polydispersity index (PdI) using a Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano ZS, Malvern Instruments, UK) equipped with a He-Ne laser at a wavelength of 633 nm. All measurements were performed at 25 °C. Each measurement of size and PdI was performed with a detection angle of 173°. The results are given as the average ± standard deviation of nine measurements.

2.4.3. Morphology

The morphology of Lf-GMP nanohydrogels coated with chitosan were evaluated by transmission electron microscopy (TEM) (EM 902A, ZEISS, Germany). TEM samples were prepared by depositing the same suspensions on a carbon-coated copper grid. Before being analysed, samples were air-dried.

2.4.4. Fourier transform infrared (FTIR) spectroscopy

In order to confirm the presence of the chitosan in Lf-GMP nanohydrogels, FTIR analyses were carried out with a Thermo Nicolet 6700 Fourier transform infrared spectrometer from Thermo-Fisher Scientific, scanning from 500 to 4000 cm^{-1}, 32 scans were collected for each sample.