Behavior of lactoferrin nanohydrogels incorporating curcumin as model compound into food simulants

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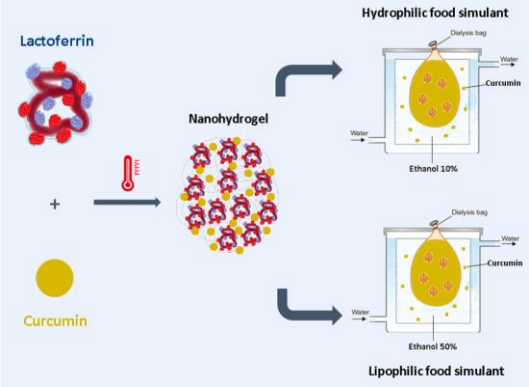

In this study, a lactoferrin (LF) nanohydrogel was developed to encapsulate curcumin as a nutraceutical model aiming at its behavior evaluation. The release kinetics of curcumin from LF nanohydrogels were also performed when added to food simulants (hydrophilic medium_ethanol 10 % and lipophilic medium_ethanol 50 %) (According to the COMMISSION REGULATION EU No 10/2011). For this purpose, the protein nanohydrogel isolated and loaded with curcumin was comprehensively characterized resorting to several techniques such as dynamic light scattering (DLS), fluorescence measurements, circular dichroism (CD), Fourier-transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). This system was able to associate curcumin at 80 μg/mL with a remarkable efficiency of ~90 % and loading capacity of ~3 %. LF nanohydrogel showed higher release rates of curcumin in a lipophilic food simulant (after ~10 hours) in comparison with a hydrophilic one (after ~25 hours).

Introduction

LF is a globular single-chain glycoprotein of the transferrin family folded into two globular lobules, which is present in several fluids such as milk, saliva, tears and nasal secretion. As one of the components of the immune system of the body, LF has great biological properties such as antibacterial, antiviral, immunomodulatory and iron binding capacity [1].

Curcumin, a yellowish polyphenol from Turmeric spice (*Curcuma Longa*), has shown to have multiple health benefits due to its anti-inflammatory and antioxidant potential. However, this nutraceutical is sparingly soluble in aqueous solutions and also presents low bioavailability [2].

Protein nanohydrogels are characterized by their three-dimensional and hydrophilic nano-sized networks coupled with their large surface area, and an interior network for incorporation of nutraceuticals, enabling: (i) their encapsulation and controlled release; (ii) their improved solubility and bioavailability; (iii) their target deliver in the associated tissues and/or protecting them against degradation and undesirable chemical reactions; and (iv) their stability in the GI tract [3].

This study is focused on the bovine LF nanohydrogel behavior as an encapsulating agent of curcumin, as well as on the release profiles of the chosen nutraceutical model when incorporated into food simulant models.

Materials and methods

Nanohydrogels preparation was based on a methodology described by other authors, with some modifications [4]. Briefly, a weighted amount of LF was dissolved in distilled water at 25 °C and stirred at 500 rpm for 1 h until reach a 0.2 % (w/v) concentration. The solution pH was adjusted to 7.0, with 0.1 mol L^{-1} of sodium hydroxide. LF aqueous solution was submitted to a thermal treatment in which it was heated at 75°C for 0, 5, 10, 15 and 20 min in a water bath (closed system), to promote the formation of a monodisperse nanohydrogel solution. All the samples were made in triplicate and kept at room temperature (25 °C) for at least 30 min until further characterization.

Initially, it was prepared a range of concentrations between 5 and 80 μg/mL from a curcumin stock solution, previously dissolved in pure ethanol. A given volume from each of these solutions was added to the LF solution and, after 20 min of gentle stirring, LF-curcumin mixtures were finally heated at 75 °C in a water bath (closed system) for 10 min. The unbound curcumin was removed from the nanohydrogel solutions by centrifuging at 12 000g for 20 min. The pellet composed by undissolved curcumin was thoroughly dissolved in pure ethanol and further quantified spectroscopically at 425 nm [5]. In order to determine the highest association efficiency (AE) for this system, the amount of free curcumin was estimated using a calibration curve previously made with the same conditions of the free curcumin solutions. Finally, the obtained values were used as the main variables in a standard AE equation.

The physicochemical characterization was carried out starting with DLS to access hydrodynamic diameter, polydispersity index (PdI) and ζ-potential of the LF-curcumin nanoparticles. Therefore, to analyze protein-nutraceutical interactions, fluorescence measurements such as ANS (1-anilinonaphthalene-8-sulfonic acid) probe and FRET occurrence analysis were performed. To evaluate the effect of curcumin association on LF secondary structure, a CD measurement was accomplished. In order to perceive the system binding types as well as to confirm LF-curcumin association, FTIR measurements were realized. TEM was also used to evaluate the morphology of nanohydrogels and to confirm the nanoparticles formation.

A release kinetics assay was performed with 2 different food simulants at room temperature (25 °C). Nanohydrogels solutions were placed in dialysis membranes with 10 kDa cut-off, which in turn those emerged into glass release reactors. Firstly, it was traced the curcumin release profile from nanohydrogels in a lipophilic food simulant (ethanol at 50%), followed by the hydrophilic food simulant (ethanol at 10%) one.

In order to evaluate Lf-curcumin nanohydrogel, the storage stability over time was performed at 4 °C and 25 °C during 35 days, separately. The stability of nanohydrogels was accessed by measuring its size, PdI and ζ -potential.

Results

LF nanohydrogel system can associate curcumin with an efficiency of 90 ± 1.09 % and loading capacity of 2.6 ± 0.02 %, for curcumin at 80 μg/mL (as shown in Table 1). Above this concentration, the system can also associate curcumin but, it

proved to be unstable, revealing a curcumin precipitate after 3 days of storage at 4 °C.

Nanohydrogels have sizes around 89.4 ± 2.2 nm with 0.197 ± 0.019 PdI and a ζ-potential of 23.4 ± 2.05 mV.

To evaluate potential hydrophobic interactions between LF and curcumin, an ANS probe extrinsic fluorescence measurement (with excitation wavelength at 370 nm) was carried out with a range of curcumin concentrations between 10 and 80 μg/mL. The results reveal that there is a curcumin-ANS competition for LF hydrophobic sites. While at the lower concentration ANS still fluoresces, at the following concentrations, the fluorescence intensity signal presents an abrupt loss, suggesting that curcumin is occupying the majority of LF hydrophobic sites. Regarding FRET analysis, results have shown that, in this system, there is energy transfer from LF tryptophan and tyrosine residues (donor fluorophores in the excited state) to curcumin chromophores (acceptor ligands in the ground state), since it can be visualized by the spectral overlap between LF fluorescence and curcumin absorbance spectra. This confirms the occurrence of FRET phenomenon, which can provide accurate structural information leading to determine protein-ligand binding distances [6].

Concerning protein secondary structure, the CD spectra shows no significant differences between native LF, LF nanohydrogels and LF-curcumin nanohydrogels. These results suggest that curcumin association does not affect LF secondary structure at a significant level.

As shown in Figure 1, the release rates of curcumin from LF nanohydrogels proved to be higher in the lipophilic food simulant rather than the hydrophilic one. It was also observed that, in the case of the hydrophilic food simulant, LF nanohydrogels nets can retain curcumin for more than over 9 days, since only ~1.6 μg of curcumin was released during this period, reaching stabilization after ~10 hours of release. On the other hand, ~16 μg of curcumin were released from LF nanohydrogels to the ethanol 50 % medium.

During 35 days, nanohydrogels showed a constant stability in terms of size, PdI and ζ-potential, at 4 °C. Solutions kept their orange-ish aspect, showing no signs of precipitate and possible contaminations. On the opposite way, after 14 days, solutions stored at 25 °C presented loss in color and it was also observed the presence of curcumin precipitates. DLS measurements showed significant changes in terms of size and PdI values, evidencing the nanohydrogels instability under such conditions.

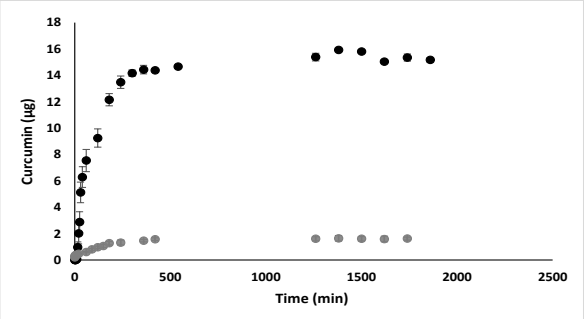

Figure 1. Release kinetics profile of curcumin from LF nanohydrogels in hydrophilic medium_ethanol 10 % (●) and lipophilic medium_ ethanol 50 % (●).

Conclusions

LF nanohydrogel has the ability to associate lipophilic nutraceuticals, such as curcumin, with remarkable association efficiency and loading capacity values. This system can also be stable over time when submitted to storage conditions (4 °C during 35 days), making it a valuable candidate to serve as vehicle for nutraceuticals controlled release.

FRET occurrence brings out the possibility to obtain valuable structural information in what LF-curcumin binding concerns.

Curcumin release rates indicate that its retention in LF nanohydrogel nets can be higher when in contact with a hydrophilic food matrix. This information suggests which matrix character would be more appropriate to incorporate LF-curcumin nanohydrogel.

Table 2. Effect of curcumin concentration on association efficiency and loading capacity of LF nanohydrogels, where data are presented as mean ± 95 % confidence interval. Different letters indicate statistically significant differences between values ($p < 0.05$).

	10 μg/mL	**20 μg/mL**	**40 μg/mL**	**60 μg/mL**	**80 μg/mL**
Association efficiency (%)	78.9 ± 2.0 % [a]	80.8 ± 4.6% [a]	84.4 ± 2.1% [ab]	85.2 ± 1.7% [ab]	90.0 ± 1.1% [b]
Loading capacity (%)	0.3 ± 0.01% [a]	0.6 ± 0.04% [b]	1.2 ± 0.06% [c]	2.1 ± 0.05% [d]	2.6 ± 0.02% [e]

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