

**Nanoencapsulation of bovine lactoferrin for oral hygiene applications**

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**INTRODUCTION**

Lactoferrin (LF) is a glycoprotein that belongs to iron transporter or transferrin family. It was originally isolated from bovine milk, where it is found as a minor protein component of whey proteins. LF contains 703 amino acids and has a molecular weight of ca. 80 kDa. In addition to its presence in milk, it is also found in exocrine secretions of mammals and is released from neutrophil granules during inflammation. It is considered a multifunctional or multi-tasking protein, and appears to play several biological roles. Owing to its iron-binding properties, LF is thought to play a role in iron uptake by the intestinal mucosa of the suckling neonate, which make it a potential source of iron for breast-fed infants. Additionally, LF showed to have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory activities (Yamauchi et al., 1993; Ward et al., 2002). Bovine LF, derived from whey proteins, is marketed as a nutritional supplement. The development of strategies that may permit structural and functional stabilization of LF via nanoencapsulation may increase the biopharmaceutical applicability of this bioactive protein. Such LF-encasing nanovesicles could be utilized in the formulation of dental elixirs to eliminate the oral microflora responsible for formation of oral biofilms with one clear advantage: the adhesive nanovesicles could slowly liberate its naturally antimicrobial content, thus reducing the development of dental cavities. Water-in-oil-in-water emulsions are examples of multiple emulsions, in which dispersions of small water droplets within larger oil droplets are themselves dispersed in a continuous aqueous phase (Bibette et al., 1999; Ficheux et al., 1998; Wang et al., 2006; Hanson et al., 2008). Emulsions are quite utilized in many ways of processing, and are extensively used by the food, cosmetic, and coating industries. Due to their compartmentalized internal structure, multiple emulsions present advantages over simple oil-in-water (O/W) emulsions for encapsulation, such as the ability to carry both polar and non-polar molecules, and a better control over releasing of therapeutic molecules (Pays et al., 2002; Davis and Walker, 1987; Okochi and Nakano, 2000). In the present research work, the potential of natural antimicrobial, bovine LF, stabilized via nanoencapsulation for incorporation in a mouth wash elixir has been investigated. Bovine LF was entrapped within water-in-oil-in-water (W/O/W) multiple nanoemulsions, aiming at mimicking the multifunctional design of biology, with several lipid matrices, and stabilizing layer compositions. The composition of the stabilizing layer of the nanosystem was changed by using different proportions of lecithin and different poloxamers (nonionic triblock copolymers with a central hydrophobic core of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene), thus allowing to control degradation times of the nanovesicles. For the production of nanoemulsions, bioactive compounds were previously dissolved in the aqueous phase prior to be dispersed in the melted lipid during homogenization in a UltraTurrax (model T25D from IKA) under heating (ca 40 °C). The resulting nanoemulsions were subsequently fully characterized physicochemically. Physicochemical characterization of the optimized nanovesicle formulations encasing the bioactive LF encompassed determination of particle size, size distribution and particle charge, via Zeta potential analysis, surface morphology via scanning electron microscopy (SEM), protein encapsulation efficiency via spectrophotometric analysis, and thermal analysis via Differential Scanning Calorimetry (DSC). The antimicrobial activity of the nanoemulsions were also assessed *in vitro*, upon several microbial strains in liquid medium, including pathogenic

bacteria and *Candida albicans*. The use of these multiple nanoemulsions in formulating elixirs for oral hygiene would possess inherent advantages, when compared with the current chemical antimicrobial formulations, in that LF is a naturally harmless protein with bacteriostatic activity, without toxicological risk even for small children.

**MATERIALS AND METHODS**

**Preparation of multiple LF-encasing nanoemulsions.** Production of multiple nanoemulsions (*water-in-oil-in-water*, W/O/W) with nanoencapsulated LF was carried out in an UltraTurrax (model T25D from IKA) under heating (ca 40 °C), with the bioactive LF being previously dissolved in the (inner) aqueous phase (W) prior to being dispersed in the melted lipid (Softisan, from Sasol Olefins & Surfactants GmbH, Hamburg, Germany) during homogenization (10 min at 8000 rpm). Therefore, final W/O/W dispersion of LF was obtained via a sequential (optimized) homogenization of a W/O dispersion involving two cycles for 10 min. The inner aqueous phase was constituted by HCl 10 mM (from Vaz Pereira), CaCl<sub>2</sub> 20 mM (from Merck), Tween 80 (from Sigma-Aldrich) and pure bovine lactoferrin (from DMV International) (with concentrations ranging from 25 mg to 250 mg); the intermediate oily phase encompassed glycerol (from Merck, Darmstadt, Germany), Softisan (or Precirol or Compritol, from GatteFossé) and soybean phosphatidylcholine (from Acofarma); finally, the outer aqueous phase encompassed Lutrol 68 (poloxamer 188 from BASF), ultrapure water and NaCl (from Vaz Pereira, Portugal) 10 mM. Control nanoparticles were also produced, without LF, and stored for 52 days under the same conditions.

**Evaluation of the combined effects of homogenization time, LF concentration and ionic strength of the outer aqueous phase.** The stability of the multiple nanoemulsions was evaluated by producing emulsions as result of the combined effect of homogenization time (5, 10 and 20 min), LF concentration (25, 50, 100 and 250 mg) and ionic strength (NaCl 1, 10 and 100 mM) of the aqueous outer phase

**Determination of hydrodynamic size and Zeta potential.** Zeta potential is the electric potential that prevails in the cutting plan of a particle, which is at a small distance from its surface, and which results from the measure of the mobility distribution of a dispersion of charged particles as they are subject to an electric field. The mobility can be defined as the velocity of a particle per unit electric field, and is determined by applying an electric field to the dispersion of particles and measuring their average velocity. The results obtained allow determination of the hydrodynamic size of the lipid vesicles, of the polydispersion index and of their Zeta potential. The Zeta potential and hydrodynamic size of the nanoemulsion particles were determined in a Zetasizer (model Nanoseries Nano-ZS) from Malvern Instruments.

**Thermal analysis by differential scanning calorimetry (DSC).** Calorimetric analysis was performed in a differential scanning calorimeter (Shimadzu, Kyoto, Japan). For every calorimetric assay, ca. 10 mg of emulsion were weighed (using a microsyringe) directly into the interior of high-pressure aluminum pans (Shimadzu, P/N 557 201-53090, 64U3670609), and duly sealed by pressure. A reference aluminum pan was also prepared by simply sealing air inside an empty case. The samples were then heated from room temperature to 100 °C at a constant linear scanning rate of 5 °C/min, during which the amount of heat absorbed by the sample was recorded.

**Protein encapsulation efficiency.** Protein encapsulation efficiency was determined via spectrophotometric analysis of the supernatants, following centrifugation of the nanoemulsions using Centricon tubes (from Millipore).

**Antimicrobial activity of LF and multiple nanoemulsions.** Screening of antimicrobial activity was determined by the diffusion method. Inocula of each target microorganism - *Staphylococcus aureus*, *Salmonella* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria innocua* and *Bacillus cereus*, and of *Candida albicans* were prepared by suspension of a pure colony in 0.9 % sterile NaCl (to reach a standard turbidimetry of 0.5 McFarland). Each suspension was inoculated in Mueller Hinton Agar, and after drying, separated wells were made in the plates, and 60  $\mu$ L of different nanoemulsion samples and controls were added. Sterile water was used as negative control, and antibiotics as positive controls (chloramphenicol for *St. aureus*, *Salmonella* sp., *E. coli*, *P. aeruginosa*, *B.s cereus* and ampicillin for *L. innocua*). Each plate was incubated at 37 °C for 24 h, after which the inhibition halos were measured. In the case of pure LF and nanoemulsion samples with antimicrobial activity, Minimum Inhibitory Concentration (MIC) was determined by microplate assay, using same target microorganisms and inocula as described previously. Each suspension of microorganism was inoculated in 96-well plates containing Mueller Hinton Broth for bacteria and Yeast Malt Broth for yeast, with different LF concentrations (2000, 1000, 500, 250, 125  $\mu$ g mL<sup>-1</sup>). Sterile water was used as negative control and uninoculated media were used as a positive control. The plates were incubated at 37 °C for 24 h, and optical density was measured by plate reader at 620 nm. The MIC was determined as the lowest concentration of LF (free or in nanoemulsion) that showed absence of turbidimetry when compared with the positive control.

## RESULTS AND DISCUSSION

The net charge of each particle surface affects the distribution of ions in the surroundings, thus forming an electrical double layer around each (nano)particle. When a particle moves, ions within the boundary move with it, and vice versa. Zeta potential is the potential that exists at this boundary, and its intensity is an indication of the potential stability in the colloidal system, and can be measured by determination of the linear velocity of the particles in an electric field. The Zeta potential depends on the concentration of ions in the solvent. The main reason why to measure the Zeta potential lies in predicting colloidal stability, which in turn depends on the interactions between particles. Zeta potential is therefore an attempt to quantify such interactions, by predicting stability. The Zeta potential is a measure of the repulsive forces between particles, and since the majority of colloidal aqueous systems is stabilized via electrostatic repulsion, the larger the repulsive forces between particles the smaller the probability for them to become closer and form aggregates, leading to a more stable colloidal system. The mean size and size distribution (polydispersity index) were measured via Dynamic Laser light Scattering (suitable for particles ranging from 5 nm to ca. 3  $\mu$ m). DSC measurements provided an insight into the state and degree of crystallinity, and the melting and crystallization behaviour of crystalline materials such as the nanovesicles produced. The amount of heat absorbed by the lipid vesicles was the same when comparing empty vesicles and vesicles incorporating LF, for a homogenization period of 10 minutes. Also, increasing homogenization time led to nanovesicles that exhibited an increase in the amount of heat absorbed by the particles, probably due to a reduction in size and/or to liberation of LF. The antimicrobial activity of the different nanoemulsions, assessed *in vitro* against several microbial strains showed different efficiency depending on the pathogenic microorganism. Buffered LF was found to be more stable than unbuffered, purified LF. Several variables were studied, viz. lipid nature, poloxamer concentration, soy lecithin concentration, homogenization time, and LF concentration. Centrifugation of the optimized nanoformulations did not lead to any LF liberation induced by bursting nanovesicles (as revealed by UV-VIS spectrophotometry), thus evidencing the firm encapsulation and concomitant structural stabilization of LF. Increasing LF concentration led to an increase in the values of Zeta potential (from negative to positive values). Optimum homogenization time was found to be 10 minutes, at 8000 rpm, since during such timeframe particle size was maintained at values ranging from 100 – 200 nm. Addition of an electrolyte

(sodium chloride) to the external aqueous phase also leads to an increase in the values of Zeta potential. However, when using a higher (100 mM) electrolyte concentration, phase separation was notorious immediately after homogenization. Increasing electrolyte concentration leads to an increase in the values of zeta potential, presumably due to accumulation of adsorbed ions at the particle surfaces. Addition of electrolyte at concentrations of 1 mM proved to be most suitable by producing nanovesicles with stable Zeta potential over time. Storage of the optimized multiple nanoemulsions (with Softisan, due to its mild melting temperature) throughout 63 d at room temperature led to an increase in Zeta potential values, presumably due to ion concentration at particle interface. Control nanoparticles were also produced, without LF, and stored for 52 days under the same conditions; for these, a slight increase in particle size was noticed, together with a slight increase in Zeta potential values. Regarding particle size, no change was observed in the range of 100 – 200 nm. Precirol and Compritol were also tested as lipids for the oily phase, but owing to their high melting points, they were discarded in order to prevent deactivation of LF.

## CONCLUSIONS

In this research effort, development and optimization of lipid nanoparticles encasing LF was pursued. A lipid with mild melting temperature was found most appropriate for the discontinuous oily phase. A homogenization timeframe of 10 min, the use of an electrolyte with a low ionic strength, and low LF concentrations were found to be critical variables for producing stable nanovesicle dispersions with diameters ranging from 100-200 nm and Zeta potential values of ca. -14.6 mV. The use of these multiple nanoemulsions in formulating elixirs for oral hygiene would possess inherent advantages, when compared with the current chemical antimicrobial formulation, in that LF is a naturally harmless protein with bacteriostatic activity.

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