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Interaction of surfactant and protein at the O/W interface and its effect on colloidal and biological properties of polymeric nanocarriers

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ABSTRACT

Hypothesis: The use of polymer-based surfactants in the double-emulsion (water/oil/water, W/O/W) solvent-evaporation technique is becoming a widespread strategy for preparing biocompatible and biodegradable polymeric nanoparticles (NPs) loaded with biomolecules of interest in biomedicine, or biotechnology. This approach enhances the stability of the NPs, reduces their size and recognition by the mononuclear phagocytic system, and protects the encapsulated biomolecule against losing biological activity. Different protocols to add the surfactant during the synthesis lead to different NP colloidal properties and biological activity.

Experiments: We develop an *in vitro* model to mimic the first step of the W/O/W NP synthesis method, which enables us to analyze the surfactant-biomolecule interaction at the O/W interface. We compare the interfacial properties when the surfactant is added from the aqueous or the organic phase, and the effect of pH of the biomolecule solution. We work with a widely used biocompatible surfactant (Pluronic F68), and lysozyme, reported as a protein model.

Findings: The surfactant, when added from the water phase, displaces the protein from the interface, hence protecting the biomolecule. This could explain the improved colloidal stability of NPs, and the higher biological activity of the lysozyme released from nanoparticles found with the counterpart preparation.

1. Introduction

Nanometer-scale biocompatible and biodegradable polymeric particles, such as those formed with polylactide glycolic acid (PLGA), are designed and optimized to carry a wide variety of biomolecules. They have been widely studied for use as drug-delivery vehicles for long-term sustained-release preparations [1,2].

Several methods are available for preparing PLGA NPs and for incorporating biomolecules into them depending on the biomolecule characteristics, the desired delivery path, and the release profile. The spontaneous emulsification solvent diffusion (SESD) method is the basis for different methods of preparing polymeric NPs. Nanosized particles can be synthesized by pouring a PLGA organic solution into an aqueous phase (or surfactant solution) with mechanical stirring and finally a solvent-evaporation process. For the preparation of NPs loaded with biomolecules, the SESD method is modified, and a double-emulsion

Abbreviations: ADSA, axisymmetric drop-shape analysis; DLS, dynamic light scattering; DMC, dichloromethane; E, dilatational modulus; EA, ethyl acetate; EE, protein-encapsulation efficiency; F68-O, procedure in which the Pluronic F68 was dissolved in the organic phase; F68-O-Lys NPs, lysozyme-loaded nanoparticles resulting from the F68-O method; F68-O-Lys5.5, F68-O-Lys9, F68-O-Lys12, lysozyme-loaded nanoparticles resulting from the F68-O method, when the pH of the lysozyme solution is: 5.5, 9.0 or 12.0; F68-W, procedure in which the Pluronic F68 was dissolved in the aqueous phase; F68-W-LysNPs, lysozyme-loaded nanoparticles resulting from the F68-W method; F68-W-Lys5.5, F68-W-Lys9, F68-W-Lys12, lysozyme-loaded nanoparticles resulting from the F68-W method, when the pH of the lysozyme solution is: 5.5, 9.0 or 12.0; i.e.p., isoelectric point; M_E, final encapsulated amount of lysozyme; M_F, total mass of lysozyme in the aqueous supernatant; M_I, initial total mass of lysozyme; $M_{polymer}$, mass of PLGA in the formulation; MPS, mononuclear phagocytic system; NPs, nanoparticles; NTA, nanoparticle tracking analysis; O/W, oil/water; PB, phosphate buffer; PDI, polydispersity index; PEO, Poly(ethyleneoxide); PL, final protein loading; PLGA, Poly(lactide-co-glycolide) acid; SEM, scanning electron microscopy; SESD, spontaneous emulsification solvent diffusion; STEM, scanning transmission electron microscopy; W/O/W, water/oil/water; μ-average, average electrophoretic mobility

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(water/oil/water, W/O/W) solvent-evaporation technique is used [3]. In this case, a biomolecule water (or buffered) solution is added onto the organic polymeric solution and mixed by mechanical energy. This first water/oil (W/O) emulsion is immediately poured into the second polar phase.

The addition of stabilizers during the preparation method, such as poly(ethylene oxide) (PEO) surfactants, is a promising way to protect the biomolecule from losing activity during its encapsulation, storage, delivery, and release [4–9]. The use of the polymeric surfactant Pluronic F68 also reduces the size of the NPs, and enhances their stability. In addition, the recognition of the nanocarriers by the mononuclear phagocytic system (MPS) is reduced.

In a previous work [10], we developed and optimized two different formulation methods for protein-loaded NPs (PLGA colloidal particles) based on the double-emulsion W/O/W solvent-evaporation technique. They differed mainly in the phase the surfactant (Pluronic F68) was added from. In both cases we obtained hard spherical NPs, but with different colloidal properties (size distribution, electrokinetic charge, colloidal stability) hence strongly influencing cellular uptake. In particular, we obtained improved *in vitro* biological activity of the released protein, and better release pattern when the surfactant was added from an aqueous phase [10]. The protein used was lysozyme, as it is considered to be a model for proteins having potential therapeutic applications (e.g. bone morphogenetic proteins) [11,12].

Accordingly, the aim in this work is to evaluate in detail how the solvent used for the surfactant and the conditions of the protein solution can affect ultimately the biological activity and colloidal stability of the NPs, by determining the protein/surfactant interactions and the interfacial composition. To this end, we analyze the properties of the protein at the interface as a function of: a) the procedure to add the surfactant (from the water or oil phase), and b) the conditions of the protein solution (pH). The interfacial results importantly correlate with the properties of the colloidal systems synthesized following the corresponding conditions and explain the different biological activity encountered depending on the method of preparation used [10].

2. Materials and methods

2.1. Formulation of the nanoparticles

Poly(lactide-co-glycolide) Acid (PLGA 50:50) ([C_2 H_2 O_2]x [C_3 H_4 O_2]y) x = 50, y = 50 (Resomer® 503 H), 32–44 kDa was used as the polymer, and polymeric surfactant Pluronic®F68 (Poloxamer 188) (Sigma-Aldrich-P7061) served as the emulsifier. The structure, based on a poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide), is expressed as PEOa-PPOb-PEOa with a = 75 and b = 30. Lysozyme from chicken egg white (Sigma-L7651) was used as a hydrophilic protein. This is a small globular protein with 129 amino acids, a molecular weight of 14,300 g/mol, and an isoelectric point (i.e.p.) of 11.35 [13].

Ultrapure water, passed through a Milli-Q water-purification system (0.054 mS), was used to prepare the buffer solutions. All glassware was washed with 10% Micro-90 cleaning solution and exhaustively rinsed with tap water, isopropanol, deionized water, and ultrapure water (in that sequence). All other chemicals were of analytical grade and used as received.

The two different formulation methods used were based on those we developed in a previous work [10]. Here, we use the terms F68-O and F68-W to designate the procedure in which the Pluronic F68 was dissolved in the organic (O) and aqueous phase (W), respectively. For both methods, the primary water/oil (W/O) emulsion has been prepared using three different pH conditions for the lysozyme buffered solution: 5.5 (water), 9 (boric acid 0.1 M), and 12 (di-sodium phosphate 0.03 M). Lysozyme presents the highest charge at pH 5.5, falling to 75% of that value at pH 9.0, both being positive. At pH 12.0 the charge turns negative with an absolute value of 38% of its value at pH 5.5 [14].

Briefly, in the F68-O method, 25 mg of PLGA, and 15 mg of Pluronic F68 were dissolved in 660 µL of dichloromethane (DMC), and vortexed. Then, 330 µL of acetone were added, and vortexed. Next, 100 µL of an aqueous buffered solution with lysozyme (5 mg/mL) were added dropwise while vortexing for 30 s. This primary W/O emulsion was immediately poured into a glass containing 12.5 mL of ethanol under magnetic stirring, and 12.5 mL of MilliQ water were added. After 10 min of magnetic stirring, the organic solvents were rapidly extracted by evaporation under vacuum until the sample reached a final volume of 10 mL.

In the F68-W method, 100 mg of PLGA were dissolved in a tube containing 1 mL of ethyl acetate (EA), and vortexed. To prepare the primary emulsion, 40 µL of a buffered solution with lysozyme (20 mg/mL) were added and immediately sonicated (Branson Ultrasonics 450 Analog Sonifier), fixing the *duty cycle* dial at 20% and the *output control* dial at 4, for 1 min with the tube surrounded by ice. This primary W/O emulsion was poured into a plastic tube containing 2 mL of a buffered solution (pH 12.0) of F68 at 1 mg/mL, and vortexed for 30 s. Then, the tube surrounded by ice was sonicated again at the maximum amplitude for the micro tip (*output control* 7), for 1 min. This second W/O/W emulsion was poured into a glass containing 10 mL of the buffered F68 solution and kept under magnetic stirring for 2 min. The organic solvent was then rapidly extracted by evaporation under vacuum to a final volume of 8 mL.

For clarity, the lysozyme-loaded nanoparticles resulting from the two methods described above are designated as F68-O-Lys and F68-W-Lys NPs. When the pH of the lysozyme solution is specified (pH 5.5, 9.0 or 12.0) we will refer to the NPs as F68-O-Lys5.5, F68-O-Lys9, F68-O-Lys12 (for the F68-O method), and F68-W-Lys5.5, F68-W-Lys9, F68-W-Lys12 (for the F68-W method).

2.1.1. Cleaning and storage

After the organic solvent evaporation, the sample was centrifuged during 10 min at 20 °C at 14,000 and 12,000 rpm for F68-O and F68-W methods, respectively. The supernatant was filtered using 100 nm filters for measuring the free non-encapsulated protein. The pellet was then resuspended in PB up to a final volume of 4 mL and kept refrigerated at 4 °C.

2.1.2. Protein loading and encapsulation efficiency

The initial protein loading was optimized for the nanoparticle formulation, preserving the final colloidal stability after the evaporation step and being different for each nanosystem. The protein-encapsulation efficiency (EE) was determined from the initial total mass of lysozyme (M_I), and the total mass of lysozyme in the aqueous supernatant (M_F), which corresponded to the free non-encapsulated protein, and was tested by the bicinchoninic acid assay (BCA, Sigma-Aldrich).

$$EE = \frac{M_I - M_F}{M_I} \times 100$$

For the final protein loading (PL), the mass of PLGA in the formulation ($M_{polymer}$) was also taken into account:

$$PL = \frac{M_I - M_F}{M_{Polymer}} \times 100$$

2.2. Characterization of the nanoparticles

2.2.1. Nanoparticle size and electrokinetic mobility

The hydrodynamic diameter and electrophoretic mobility of the NPs were determined by using a Zetasizer NanoZeta ZS device (Malvern Instrument Ltd, U.K.) working at 25 °C with a He-Ne laser of 633 nm, and a 173°scattering angle. Each data point was taken as an average over three independent sample measurements. Dynamic Light Scattering (DLS) was used to determine the average hydrodynamic diameter (Z-average or cumulant mean), and the polydispersity index