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Physico-chemical studies of molecular interactions between non-ionic surfactants and bovine serum albumin

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This paper is dedicated to the memory of Prof. Simón Rodríguez-Calvo.

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ABSTRACT

Surfactants, particularly non-ionic types, are often added to prevent and/or minimize protein aggregation during fermentation, purification, freeze-drying, shipping, and/or storage. In this work we have investigated the interactions between two non-ionic surfactants (Tween 20 and Tween 80) and bovine serum albumin (BSA), as model protein, using surface tension, fluorescence measurements and computational analysis. The results showed that, in both cases, the surface tension profile of the surfactants curve is modified upon addition of the protein, and the CMC values of Tween 20 and Tween 80 in the presence of protein are higher than the CMC values of the pure surfactants. The results indicate that although Tween 20 and Tween 80 do not greatly differ in their chemical structures, their interactions with BSA are of different nature, with distinct binding sites. Measurements at different protein concentrations showed that the interactions are also dependent on the protein aggregation state in solution. It was found from fluorescence studies that changes observed in both the intensity and wavelength of the tryptophan emission are probably caused by modifications of tryptophan environment due to surfactant binding, rather than by direct interaction. Based on a computational analysis of a BSA three-dimensional model, we hypothesize about the binding mechanism of non-ionic surfactant to globular protein, which allowed us to explain surface tension profiles and fluorescence results.

1. Introduction

Protein pharmaceuticals are subjected to a number of stresses during production, storage, and shipping, resulting in loss of the protein concentration and activities or formation of soluble and insoluble aggregates. The general method for stabilizing liquid protein pharmaceuticals is the use of formulation excipients. Although surfactants have been commonly used to stabilize proteins, the mechanism of their action has not been fully explained. The dual nature in its amphiphilic structure causes surfactants to adopt specific orientations at interfaces and in aqueous solutions, and this is usually the characteristic that lies at the root of the mechanisms by which surfactants affect the physical stability of proteins. Moreover, their stabilizing effects have empirically shown to be concentration- and protein-dependent, although high concentrations of surfactant may not be necessarily more effective, and in some cases, can have negative effects [1]. It becomes evident that to efficiently design stable protein formulations we need a more comprehensive understanding of the interactions of proteins with the various components of a formulation and their effect on protein stability [2].

There are many reports in the literature on specific effects of surfactants on proteins showing that they can be both stabilizing and destabilizing to protein structure, this latter effect being more common at high concentrations. Therefore, it is desirable to minimize the addition of surfactants when using them as excipients in a pharmaceutical formulation containing proteins. In this sense, it has been established that any insights on the mechanism(s) by which a particular protein is protected from damage by surfactant addition is relevant in the development of these pharmaceuticals [3].

In general, surfactant–protein interactions are not well understood, and most comprehensive studies use ionic surfactants (e.g. sodium dodecyl sulfate, SDS) since interactions are stronger and interpretation of results is somehow easier [4,5]. However, the surfactants that are normally used in formulations are non-ionic, for their stabilizing properties, while ionic surfactants can also bind to

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oppositely charged polar groups in proteins and cause denaturation [3]. In particular, physico-chemical studies on surfactants used for pharmaceutical formulations are needed, since most reports are focused on their use as protein denaturing agent, on their effect on the competitive adsorption at fluid interfaces, and on their relevance in the stabilization of emulsions and foams in the food industry.

Similarly to polymers, most proteins do not associate with non-ionic surfactants, with the general exception being those containing hydrophobic pockets or patches, such as serum albumins. The hydrophobic portion of non-ionic surfactants can bind to hydrophobic patches on proteins, which naturally causes the surfactant to order itself so that more hydrophilic groups are solvent exposed. Consequently, the surfactant:protein complex becomes more hydrophilic than either the surfactant or the protein alone, and the effective increase of the complex solubility avoids formation of higher order aggregates [3]. Adsorption of surfactant and protein molecules in mixed systems is competitive. For example, non-ionic surfactants usually bind tighter than proteins or surfactant:protein complexes at interfaces [6]. Thus, above a critical concentration of the surfactant, protein adsorption becomes negligible and the adsorption isotherms for mixed surfactant/protein systems can be roughly identical to that of a pure surfactant solution [7].

In addition to altering the interaction of proteins with surfaces, non-ionic surfactants can also interact directly with proteins in solution. Polyoxyethylene sorbitan monolaurate (Tween 20) has been reported to act as a chemical chaperone, aiding in the refolding of proteins via hydrophobic interactions [2,3]. Nevertheless, it has also been reported that in some cases non-ionic surfactants have no effects on the protein stability [8].

Low concentrations of non-ionic surfactants are often sufficient to prevent or reduce protein surface adsorption and/or aggregation due to their relatively low critical micelle concentration, CMC (10^{-6}–10^{-5} mol L^{-1}) [2]. This is due to the high surface-activity of this class of excipients, which renders a higher effective concentration of surfactant molecules at interfaces than in bulk solution. However, this property makes it more difficult to study their interaction with proteins, since it is difficult to obtain and interpret data at these low surfactant concentrations. In general, surfactant–protein interactions can be studied using various indirect methods, e.g. tensiometry [5,9–11], conductivity [12–14], viscosity [5–11], and spectroscopic techniques [11,15–18], as well as by more direct measurements such as dialysis [19,20] and ion-selective electrodes [21,22]. However, most of these techniques are used for ionic surfactants, due to the easiness and variety of measurement and interpretation of results, and only a few reports are devoted to their application as excipients in pharmaceutical formulations [3,23,24].

In this work we used tensiometry, fluorescence spectroscopy and computational analysis to evaluate the surfactant–protein interactions, by studying the effect of non-ionic surfactants of the polyoxyethylene sorbitan family (Tween 80 and Tween 20, a.k.a. polysorbates) on a well-known globular protein, namely bovine serum albumin (BSA). Polysorbates are a group of non-ionic surfactants that find widespread use as emulsifiers, defoamers, dispersants, and stabilizers in food, cosmetics, and pharmaceutical formulations. Typically, due to their production process, the commercial product is a complex mixture. MALDI-TOF mass spectrometric studies of polysorbate formulations have revealed a complex mixture of oligomers that include polyethylene glycol esters, sorbitan polyethoxylates, polysorbate diesters, and sorbitol polyethoxylate esters, where the major component of the fatty acids esters of polyethoxy sorbitan defines the name of the product [25,26]. This diversity of chemical structures in the commercial products makes the physico-chemical studies and the interpretation of results more difficult. At the same time, a systematic approach to Tween–protein interactions is needed in order to optimize the use of these excipients in protein pharmaceuticals, since their efficacy is probably due largely to their heterogeneity [26]. It is also our aim in this report to emphasize on the importance of considering the specific properties of these surfactants, and the consequences that apparently small structural differences can have on the interactions responsible for the stabilization of proteins in aqueous formulations.

2. Material and methods

2.1. Reagents

Bovine Serum Albumin (BSA, 66 411 g mol^{-1}), polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Merck and used without further purification. Aqueous stock surfactant solutions were prepared one day in advance to ensure full hydration of micelles. Protein solutions were freshly prepared in water before performing analysis. The protein concentration was determined by spectrophotometry using an extinction coefficient (ε_{280}) of 44 720 L mol^{-1} cm^{-1} [27]. Typical buffers used in pharmaceutical formulations were supplied by the Center of Molecular Immunology (Havana, Cuba) and consisted in dibasic and monobasic sodium diphosphate and sodium chloride (phosphate buffer, pH ~6.8–7.3), and sodium citrate, citric acid and sodium chloride (citrate buffer, pH ~6.8–6.95). Distilled and Milli-Q water were used for all experiments, with specific conductivity within 1–4 μS cm^{-1} and pH ~6.5–7. All other reagents were analytically pure (Merck or BDH). A stock solution of the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (1,8-ANS) was prepared in Milli-Q water.

2.2. Surface tensiometry

Surface tension measurements of individual surfactants and protein, as well as of surfactant:protein mixtures, were performed at 298 K by the Wilhelmy method using a tensiometer by Nima Technology Ltd (U.K.), with an instrumental error of 0.1 mN m^{-1}. Surface tension (γ) measurements provided the determination of the critical micellar concentration (CMC) values in surfactant solutions, detected as the breakpoint in the γ vs. log C_{surf} plot [28]. In the case of commercial surfactants containing surface-active impurities, such as Tween 20 and Tween 80, the surface may become saturated with highly surface-active molecules, although the actual onset of micellization may take place at a higher surfactant concentration [29]. Therefore, to ensure consistency in the determination of CMC, critical micellar concentration values were obtained as the intersection of the two linear fits relative to the surface tension depression just before the CMC and the further plateau.

In order to assess the reliability of CMC determination using titration, experiments were also performed in batch mode. Different sets of surfactant:protein mixtures were prepared at different ratios resembling the titration conditions. Surface tension measurements were done immediately after mixing and 24 h later. The results showed that although the determination of CMC was easier after a longer equilibration time, due to an improvement in the break point detection, the CMC value was the same using both methods (titration vs. batch), and the mixed aggregates formed are stable in the time period studied. Experiments were done both in buffer solutions and in water.

2.3. Fluorescence measurements

Steady state fluorescence measurements were performed using the Photon Technology International (PTI) QM-1 fluorescence sys-