European Journal of Pharmaceutics and Biopharmaceutics 119 (2017) 396–407

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

The film tells the story: Physical-chemical characteristics of IgG at the liquid-air interface

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ARTICLE INFO

Article history:
Received 16 March 2017
Revised 15 May 2017
Accepted in revised form 13 July 2017
Available online 22 July 2017

Keywords:
Protein stability
Liquid-air interface
Interfacial film
Surface pressure
Surface spectroscopy

ABSTRACT

The presence of liquid–air interfaces in protein pharmaceuticals is known to negatively impact product stability. Nevertheless, the mechanisms behind interface-related protein aggregation are not yet fully understood. Little is known about the physical–chemical behavior of proteins adsorbed to the interface. Therefore, the combinatorial use of appropriate surface-sensitive analytical methods such as Langmuir trough experiments, Infrared Reflection-Absorption Spectroscopy (IRRAS), Brewster Angle Microscopy (BAM), and Atomic Force Microscopy (AFM) is highly expedient to uncover structures and events at the liquid–air interface directly. Concentration-dependent adsorption of a human immunoglobulin G (IgG) and characteristic surface-pressure/area isotherms substantiated the amphiphilic nature of the protein molecules as well as the formation of a compressible protein film at the liquid–air interface. Upon compression, the IgG molecules do not readily desorb but form a highly compressible interfacial film.

IRRA spectra proved not only the presence of the protein at the interface, but also showed that the secondary structure does not change considerably during adsorption or compression. IRRAS experiments at different angles of incidence indicated that the film thickness and/or packing density increases upon compression. Furthermore, BAM images exposed the presence of a coherent but heterogeneous distribution of the protein at the interface. Topographical differences within the protein film after adsorption, compression and decompression were revealed using underwater AFM.

The combinatorial use of physical–chemical, spectroscopic and microscopic methods provided useful insights into the liquid–air interfacial protein behavior and revealed the formation of a continuous but inhomogeneous film of native-like protein molecules whose topographical appearance is affected by compressive forces.

1. Introduction

Protein pharmaceutics are among the fastest growing and most important molecules in diagnostics and therapy, and therefore are of significant importance in high-impact areas such as autoimmune diseases and cancer [1]. The large size, the compositional variety and the distinct three-dimensional structure of protein molecules are causal for their sensitivity to undergo degradation processes.

Proteins undergo both chemical and physical degradation such as oxidation and hydrolysis, denaturation and aggregation [2]. Whereas a chemical instability reaction leads to a change in the primary structure of the protein, physical instability reactions result in a change of the spatial arrangement of the protein structure, without modification of covalent bonds. The immunogenic potential of protein pharmaceutics is directly related to the emergence of aggregates [3]. So, the maintenance of the native conformation is essential for both the efficacy, as well as the safety of a protein drug [4–7].

Protein aggregation is highly undesirable due to the profound impact on the stability of the drug product, which can result in a loss of activity and unwanted immunogenic responses. In order to control protein aggregation, it is important to understand the underlying mechanisms. The fundamentals of protein aggregation were first described in the 1960s by the Lumry–Eyring model and are continually developed further [8–11].

Under physiological conditions, the three-dimensional structure of a protein represents an equilibrium between native and denatured (unfolded) states [8,12–14]. Exogenous influences during production, storage and transportation can lead to a shift in this balance. In the unfolded state, hydrophobic patches, usually

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http://dx.doi.org/10.1016/j.ejpb.2017.07.006
0939-6411/

buried in the core, can be exposed to the outside of the molecule, and therefore the denatured proteins are more prone to aggregation. The protein aggregates can be soluble or insoluble in nature, can be composed of covalent and non-covalent bonds, and can be reversible or irreversible [15]. Moreover, not only (partially) unfolded, but also native conformations are involved in the formation of aggregates [16]. In particular, the formation of so-called "large native-like" particles often occurs spontaneously, and no continuous pathway from monomer to dimer and then to large particles can be observed [10]. For instance, a self-association of native protein molecules has been reported for highly concentrated protein solutions as a result of macromolecular crowding-effects [17].

The propensity of protein molecules to accumulate and therefore concentrate at phase boundaries (e.g. solid–liquid, liquid–liquid, and liquid–air) plays an important role in several technological processes, for example during manufacturing and storage of protein pharmaceuticals. The migration of proteins from a bulk phase to an interface is similar to the adsorption process of small amphiphilic solutes, e.g. surfactants. A major distinction, however, is that a small surfactant molecule contains a defined hydrophilic head and hydrophobic tail that can easily partition towards the aqueous and non-aqueous regions of the interface, respectively. Such straightforward partitioning is not possible in the case of proteins. While most of the hydrophilic residues in the tertiary structure of proteins are exposed on the surface, not all hydrophobic residues are buried in the interior and some of them are exposed on the surface what finally imparts amphiphilicity to protein molecules [18]. Therefore, protein molecules adsorb to the liquid–air interface and thereby do not only lower interfacial tension but also form continuous gel-like films of highly concentrated protein via mainly non-covalent interactions [19,20]. The substantial differences in the surface activity of various proteins must be therefore related to their physical, chemical and conformational properties. Apart from intrinsic molecular factors, surface activity is also dictated by several extrinsic factors, such as pH, ionic strength, temperature or presence of other solution components such as sugars or surfactants. In addition to that, the molecular size of globular proteins affects their adsorption to the liquid–air interface [21,22].

Film formation and interfacial protein gelation have been identified as important triggers for the aggregation of protein pharmaceuticals [23–27]. For instance, adsorption of proteins to silicone oil, such as in prefilled syringes, can enhance protein aggregation [28]. Moreover, particle shedding from silicone tubings in peristaltic dosing pumps has to be considered [29]. Protein aggregation is also known to occur under different mechanical stress conditions, such as shaking [30,31]. Eliminating the liquid–air interface by removing the headspace in vials prevents agitation-induced aggregation as shown by Kiese et al. [32]. Furthermore, several studies suggest a clear connection between the disruption of the highly concentrated protein layer at the liquid–air interface and the occurrence of protein particles in the bulk solution [33,34]. Choosing appropriate formulation conditions, such as pH, ionic strength and additives (e.g. non-ionic surfactants), can stabilize proteins in pharmaceutical parenteral products against adsorption at surfaces and interface-induced aggregation [35–38].

In this study, different surface-sensitive analytical methods were applied for the characterization of important functional properties, such as adsorption, compressibility, as well as structural and topographical features of interfacial protein films. The combinatorial use of different physical–chemical methods enables comprehensive insights into the protein behavior at the interface. These new findings will not only help to understand how protein stability is affected by the events happening at the interface, but also to identify and localize liquid–air interface related mechanisms of aggregation. Examination of particle formation by liquid–air interfacial stress only is not the subject of this study, but was addressed in separate investigations which are to be published soon.

2. Materials and methods

2.1. Materials

Human IgG (Beriglobin™, CSL Behring GmbH, Germany) was used for this study. The market product contains 159 mg/mL human IgG in 22 g/L Glycine and 3 g/L NaCl buffer at pH 6.8. Glycine-NaCl buffer was prepared using highly purified water (ELGA LC134, ELGA LabWater, Germany) and pH was adjusted adding NaOH. All diluted solutions were prepared by the addition of Glycine-NaCl buffer at pH 6.8 to the human IgG stock solution followed by filtration using 0.2 μm sterile PES filters (Sterile Syringe Filter PES, VWR, Germany).

2.2. Surface pressure measurements

Surface activity was expressed by surface pressure Π, with $\Pi = \sigma_0 - \sigma$, where σ_0 and σ are the aqueous subphase surface tension and the surface tension of the aqueous protein solution, respectively. Surface pressure measurements were performed in a $5.9 \times 39.7\ cm^2$ PTFE Langmuir trough equipped with a metal alloy dyne probe (Microtrough XS, Kibron Inc., Finland). For the determination of equilibrium surface pressures a 3×6 Multiwell Plate (V = 0.8 mL) was used. Results are given as mean (n = 3) and standard deviation. Equilibrium adsorption pressure is defined as the maximum surface pressure that is reached by adsorption only and stable in a range of ±0.2 mN/m within 0.5 h.

160 mL sample solution was filled into the trough for the repeated compression-decompression measurements. The surface area of the trough can be varied by two movable PTFE barriers. Temperature was kept at 20 °C (K6-cc circulation thermostat, Peter Huber Kaeltemaschinenbau GmbH, Germany). Compression speed was set to 55 mm/min, and compression-decompression cycles were conducted from a maximum surface area of $A_{max} = 210\ cm^2$ to $A_{min} = 52\ cm^2$. Compression was started after the equilibrium adsorption pressure was reached.

2.3. FT-IR spectroscopy

For FT-IR measurements spectra were recorded using a Tensor 27 (Bruker Optics GmbH, Germany) connected to a thermostat (DC30-K20, Thermo Haake GmbH, Germany). For each measurement, the protein was formulated at 10 mg/mL in Glycine-NaCl buffer pH 6.8, and for each spectrum 100 absorbance scans were collected at a single beam mode with a resolution of 4 cm^{-1}. Spectra were analyzed by Opus 7.5 (Bruker Optics GmbH) and displayed as vector-normalized second-derivative spectra (calculated with 17 smoothing points according to the Savitzky-Golay algorithms [39]). Infrared spectra of the protein in solution were recorded using an AquaSpec (transmission cell H_2O A741-1) and BioATR (Attenuated Total Reflectance) cell™ II or BioATR (Attenuated Total Reflection), respectively, at 20 °C.

Infrared spectra of the temperature-induced unfolding of the IgG samples were conducted using the BioATR cell, as this sample cell can analyze protein samples either in solution or in suspension. Reference spectra were recorded under identical conditions with only the buffer (Glycine-NaCl buffer pH 6.8) in the cell. Temperature-dependent spectra were acquired every 4 °C from 25 to 93 °C with an equilibration time of 120 s. Recorded infrared spectra were analyzed by Protein Dynamics in Opus 7.5.