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Understanding interactions between immunoassay excipient proteins and surfactants at air–aqueous interface

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ABSTRACT

Air–aqueous interfacial properties of four excipient proteins commonly used in immunoassay reagent formulations were studied with shear rheology and surface characterization methods. A *Du Noüy ring* geometry was utilized to quantify the elastic (G') and viscous (G'') shear moduli of protein interfacial networks and to probe the effect of several nonionic surfactants at various concentrations. Time sweep protocols of buffered protein solutions yielded G' in the range of 16 mN/m for bovine serum albumin (BSA), 6 mN/m for bovine gamma globulin (BGG), 7 mN/m for Mouse IgG, and 0.9 mN/m for sodium caseinate. G's were higher than G''s for a given protein. Effect of nonionic surfactants on G' of a protein was concentration dependent and the magnitude of protein displacement from the interface varied with Tween 20 > Triton X-100 > Triton X-405, with the exception of Mouse IgG. Degree of displacement of BSA from the interface by Tween 20 was approximately 66-fold greater than that of BGG whose displacement by Tween 20 was approximately 7-fold greater than that of Mouse IgG. Degree of displacement by Triton X-100 was comparable in case of studied proteins. Surface tension characterization suggests that the interfacial interactions between proteins and surfactants are driven not only by their surface activity but also by the network formation abilities of the proteins. Data presented here demonstrates a potential application of interfacial studies to sensitively identify discriminatory interactions between proteins and surfactants in immunoassay solutions.

1. Introduction

Today's clinical laboratories widely utilize immunoassay reagents for detection and quantitation of biological markers and drugs contained in patient samples such as serum, plasma, whole blood, and urine. The *in vitro* diagnostic markers vary in chemistry and form, and may constitute simple to complex proteins, lipids, steroids, and many other variable structures with interesting biochemical–physical properties [1,2]. Immunoassay reagents are commonly formulated for optimal performance with constructs of capture and luminescent detection entities dispersed in solutions of excipients such as buffers, proteins, surfactants, and other specific additives [3]. Excipient proteins usually are added into the immunoassay reagent formulations to impart better stability to the latter and prevent non-specific binding (NSB) events of biological markers or drugs [4]. Surfactants, both nonionic and ionic, are part of immunoassay reagent formulations to prevent NSB and for improved assay performance such as sensitivity and specificity [3]. Hence, understanding physico-chemical interactions between proteins and surfactants that are commonly used in *in vitro* diagnostics reagents, in bulk phase and at interfaces, may lead to reagent performance improvements. Further comprehension of these interactions and the relative magnitude of hydrophobic vs. electrostatic forces between the proteins and surfactants would certainly lead to better choice of reagent components to improve reagent stability, prevent non-specific binding, prevent protein denaturation and losses during formulation processing, and improve specificity for target markers. Experimental findings in this report constitute a step in the direction of designing improved immunoassay reagent formulations.

Proteins are flexible biological constructs made up of both hydrophobic and hydrophilic domains. In an aqueous medium, natural structural arrangement of a protein hides the hydrophobic domains from the aqueous bulk, while exposing the hydrophilic domains to it. At an interface such as air–liquid, solid–liquid, or oil–liquid, a protein molecule re-arranges itself to expose the hydrophobic regions to air, solid, or oil respectively and shield the hydrophilic regions. In case of most of the proteins, this structural rearrangement for minimizing interfacial energy leads to unfolding of proteins at interfaces followed by formation of protein films or networks via intermolecular hydrogen bonding interactions. Behavior of proteins at hydrophobic–hydrophilic interfaces has been of scientific interest to understand their impact on emulsion

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and foam stabilities in food hydrocolloids [5–8], on development of pharmaceutical formulations [9–15], and in relation to biomaterials studies [16–20]. However, only a few reports relate protein interfacial properties to immunoassay applications [21]. Characterization techniques such as interfacial shear and dilatational rheology have been widely utilized to study viscoelastic behavior of protein networks at air–liquid interfaces [20,22–36], but none of this work at this interface has been, to the best of our knowledge, driven to answer questions of direct implication in the immunoassay formulation field.

Low molecular weight surfactants owing to the presence of distinct hydrophilic and hydrophobic domains have greater surface activity than proteins [8,9,24,37]. This leads to kinetically and thermodynamically favored faster adsorption of surfactants at interfaces and potential displacement of pre-adsorbed proteins from interfaces as governed by surfactant type and concentration. Competitive displacement of proteins by surfactants and biological surface active materials from hydrophobic–hydrophilic interfaces has been characterized using techniques such as surface tension measurement [26,37–39], pendant drop/bubble tensiometry [26,40], Langmuir–Blodgett deposition followed by Atomic Force Microscopy [19,25–27,36,40,41], Brewster Angle Microscopy [37,42], interfacial shear rheology [28,42–44], total internal reflection fluorescence and ellipsometry [9], and front-face fluorescence spectroscopy [45]. Mackie et al. [25,27], Woodward et al. [36,40], and Gunning et al. [41] have reported that the competitive displacement of milk proteins β-lactoglobulin, sodium caseinate, β-casein, or mixtures of β-lactoglobulin and β-casein by nonionic surfactants, polysorbate (i.e. Tween) 20 or 60 as well as ionic surfactants, sodium dodecyl sulfate (SDS) or cetyltrimethylammoniumbromide (CTAB) from hydrophobic interfaces follow "Orogenic" displacement mechanism. Orogenic displacement, in case of nonionic surfactants, involves nucleation and growth of surfactant domains, followed by breakdown of the protein networks, and subsequent transfer of proteins into solution bulk [38,46]. Reduction in surface elasticity of weaker interfacial networks of fibrinogen, a plasma protein, in presence of anionic fluorinated and hydrogenated surfactants (C_8FONa, C_8HONa and C_{12}HONa) was observed by Hassan et al. [19]. The authors also noted that greater interaction of C_8FONa with fibrinogen resulted in formation of more surface active protein–surfactant complex, whereas negligible interactions between C_8HONa and C_{12}HONa with fibrinogen lead to protein folding. Engel et al. [47] observed that native properties of a protein, specifically bovine α-lactalbumin, could be restored by its displacement from hydrophobic surface using sufficient concentrations of nonionic Tween 20 and zwitterionic CHAPS [or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate].

In this report, we have compared viscoelastic behavior at air–liquid interface of four of the most commonly used immunoassay excipient proteins, bovine serum albumin (BSA, 66 kDa), bovine gamma globulin (BGG, 150 kDa), mouse immunoglobulin G (Mouse IgG, 150 kDa), and sodium caseinate (720 Da), before and after addition of nonionic surfactants, Triton X-100, Triton X-405 (both linear octylphenol ethoxylates), and more branched polyoxyethylene sorbitan monolaurate (or Tween 20) into equilibrated protein solutions. Molecular structures of the surfactants are shown in Supporting Information Fig. SI-F2. BSA [23], BGG [48,49], and Mouse IgG are serum proteins with predominantly globular structure. Sodium caseinate is a milk protein containing α_{s1}-, α_{s2}-, β-, and κ-casein fractions [50], and exhibits surface activity and interfacial network formation abilities similar to that of respective constituent proteins [36]. Shear rheology characterization at air–liquid interface was performed using *Du Noüy ring* geometry on an AR-G2 Rheometer, and *Wilhelmy plate balance* on a Langmuir trough was used for surface tension characterization. *Du Noüy* ring method has in the past been used extensively for surface tension characterizations [22,25,35] and recently for interfacial shear rheology [51,52]. We have employed these two techniques to characterize differences in protein–surfactant interactions at the air–liquid interface and address questions of significance to immunoassay formulation: which NSB blockers form the strongest protein interfacial networks, where does an immunoglobulin rank among these excipient proteins when tested at their respective commonly used concentrations, and finally, how does the displacement of these proteins from the air–liquid interface compare upon exposure to low levels of nonionic surfactants?

2. Materials and methods

Materials used and the preparation of protein and surfactant solutions have been described in details in the Supplementary Information section. Given below are descriptions of the methods used.

2.1. Interfacial shear rheology measurements

TA Instruments' controlled stress, direct strain, and controlled rate AR-G2 Rheometer, with accompanying Rheology Advantage data analysis software, was used for interfacial rheology characterization using *Du Noüy* ring geometry. The ring is a 10 mm diameter Pt–Ir wire ring of 0.36 mm ring thickness and was supplied by TA Instruments Waters LLC (New Castle, DE, USA). The ring was positioned at a fixed back-off distance of 45,000 μm from the base of a petri dish using instrument control software. 50 mL of a protein solution was carefully poured into a dip glass petri-dish without formation of bubbles. Bubbles, if formed, were aspirated out immediately. 50 mL sample volume reaches a freestanding height of 12.9 mm in the petri dish. The ring was initially lowered to a predefined gap of 19,000 μm using the software. As recommended in the manufacturer's product application notes, the ring was then manually brought in contact with the liquid allowing complete wetting and positioned into the plane of the liquid surface. This position of the air–aqueous interface was used as the position of the ring for all measurements. An oscillatory sweep was commenced within less than one minute of the contact between the ring and the liquid. Oscillatory time sweeps were performed at a fixed angular frequency of 0.1 Hz and amplitude of 0.5% strain – a value within the linear viscoelastic region as determined by strain sweeps – to understand formation of protein networks.

Preliminary time sweeps were conducted to obtain time duration required for a protein to attain equilibrium, at which the viscoelastic moduli of the protein network remained relatively unchanged (i.e. <10% change) over a period of approximately 30 min. Once the network reached equilibrium, required amount of Protein–Surfactant Mix to achieve a target bulk surfactant concentration was injected using a pipette at the bottom of petri-dish. This approach was used since we were interested in monitoring surfactant impact based on an order of addition that more closely mimicked the order of addition that an immunoassay reaction will follow (i.e. patient sample followed by reagent). Without removing it and creating bubbles, the pipette was rinsed with the in-use protein solution via upward–downward motion of pipette piston to generate sufficient turbulent mixing to accelerate the dispersion of surfactant throughout the protein solution. Another time sweep measurement was commenced immediately to obtain rheological behavior of the protein in presence of a surfactant. This time sweep was continued until the viscoelastic moduli remained relatively unchanged. Time sweeps were conducted for bulk surfactant concentrations of 0.001–0.01% of both Triton X-100 and Tween 20, and 0.005–0.05% of Triton X-405. Data were collected at 1 min intervals.