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Jinjiang Li
Mary E. Krause
Raymond Tu *Editors*

Protein Instability at Interfaces During Drug Product Development

Fundamental Understanding, Evaluation, and Mitigation

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Preface

Over the last two decades, the landscape of medicine has changed significantly, as biologic therapeutics have played an increasingly pivotal role in disease treatment. The recent emergence of protein-based immuno-oncological therapeutics has altered the treatment pathway for many cancer patients, providing new hope in various diseases with high mortality rates. The discovery and development of biological therapies for many diseases, such as cancer and diabetes, have been the focus of numerous international pharmaceutical and biotechnological companies, and the development of biologics frequently outpaces that of small molecule drugs. Unlike small molecule drug candidates, the therapeutic function of proteins relies not only on the primary structure (sequence) but also on the precisely folded higher order structure. Disruption of these higher order structures could result in the loss of therapeutic functions and, thus, the loss of efficacy. Therefore, during the manufacturing and formulation of biologics, the preservation of the often-fragile higher order structure of proteins is critical. Because proteins are typically surface-active, they tend to adsorb to interfaces, potentially leading to changes in conformation and denaturation. During the development and manufacturing of biologics, proteins are subjected to interfacial stresses that are present from the early-stage isolation of drug substances through eventual storage and clinical administration.

When discussing the scope of this book, we understood that the breadth of topics relevant to the scalable production and distribution of biologic drug products was wide-ranging. As a consequence, this book examines the fundamental understanding of the interfacial behavior of proteins as well as the applications of interfacial stresses in drug development. Contributions from both academicians and industrialists are folded together to illustrate how the interfacial behavior of these complex drug products can be effectively modeled and how existing protocols in industry can be used to mitigate unwanted aggregation and precipitation. Taken together, the collected sections define the impact of these interfacial stresses in the pharmaceutical development and manufacturing of biologics for an audience that should include graduate students who are hoping to enter this field and professionals who want a

deeper understanding of the role of interfaces in drug development. Additionally, we believe that the publication of this book will catalyze additional research in this area.

We thank our authors for taking their precious time to contribute this book. Additionally, we are indebted to all the reviewers of the various chapters, including Atul Bhangale, Danny Chow, Daan Crommelin, Bart Demeule, Frank Etzler, Archana Jaiswal, Cavan Kalonia, Ajit Narang, Bjorn Peters, Ronak Shah, and Joseph Zasadzinski. Finally, we are grateful to the staff of Springer for their professional expertise and guidance.

Bridgewater, NJ — Jinjiang Li
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Contents

Overview of the Impact of Protein Interfacial Instability on the Development of Biologic Products

Jinjiang Li, Mary E. Krause, and Raymond Tu

Abbreviations

DF	Diafiltration
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
MFI	Micro-flow imaging
PAT	Process analytical technologies
QCM-D	Quartz crystal microbalance with dissipation monitoring
SEC	Size exclusion chromatography
UF	Ultrafiltration

Introduction

Biologics, including protein therapeutics, cell therapy, and oligonucleotide therapeutic agents, have become an important pillar to treat diseases in the healthcare industry [1]. In the past 10 years, sales of biologic products grew significantly. In 2016, the market share for biopharmaceuticals was $266 billion, and this market share is expected to reach $399 billion by 2025 [2]. Particularly, in the area of cancer

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treatment, antibodies have become an indispensable treatment option [3]. Unlike small molecule pharmaceutics, biologics, especially protein therapeutics, need to maintain their native structure to maintain their therapeutic function [4]. Thus, physical instability of proteins is of great concern in drug product development and manufacturing [5]. Maintaining the physical integrity of proteins becomes a critical part of the manufacturing and formulation processes.

The thermodynamic transition from the protein folded state to the unfolded state requires little energy, and proteins can readily denature at interfaces, leading to formation of aggregates and particles [6–8]. Formation of aggregates and particles can result in serious consequences, including immunogenicity and the loss of efficacy. The overall development paradigm for a biologic product includes manufacturing of drug substance and subsequent conversion to drug product (filtration, filling into the final container closure system, and packaging). The details are shown in Fig. 1.

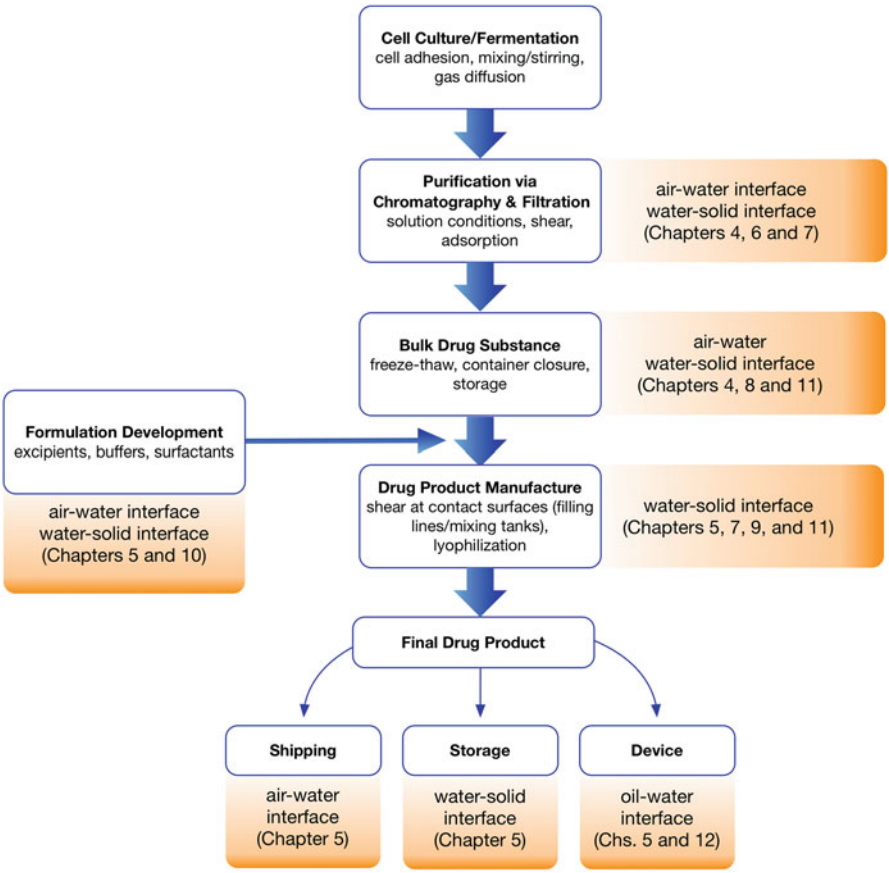

Fig. 1 Overview of drug substance and drug product manufacturing operations, with emphasis on interfacial phenomena

The drug substance manufacturing process includes upstream and downstream processing. In the case of a protein, for example, upstream processing includes expression of the target molecule, while downstream processing involves the isolation and purification of the target molecule. During downstream processes, proteins are exposed to various interfacial stresses; the target proteins are intentionally exposed to various hydrophilic/hydrophobic surfaces (i.e., chromatography resins) in order to separate them from host cell proteins and other impurities. After obtaining drug substance with the desired attributes, proteins are moved to drug product development.

During drug product development, the compatibility of proteins with both manufacturing components and container closure systems is investigated. Drug product manufacturing unit operations induce additional interfacial and shear stresses on the protein solutions. For transportation and storage, biologic drug substances are often frozen and subsequently thawed for drug product development and manufacturing. During these freeze-thaw cycles, proteins are subjected to interfacial stress through ice crystallization. Finally, in an effort to enhance patient convenience, in-clinic or in-home delivery using prefilled syringes, auto-injectors, and patch pumps are becoming preferred options for many protein-based therapies. Each of these in-home delivery modes has the potential to introduce new interfacial stresses that can denature biologics. In the following text, we will discuss the development process used in the manufacture, formulation, development, and storage of biologics, specifically focusing on the role of interfaces.

Interfaces in Product Development

Three types of interfaces are encountered in the development of biologic products: air-liquid, liquid-liquid, and liquid-solid. At the air-water interface, which is a common interface encountered in the manufacturing of drug substance and drug product, proteins often adsorb to the interface and may undergo conformational changes, followed by denaturation and/or aggregation. Both denaturation of biologics and formation of aggregates potentially cause immunogenicity and the loss of efficacy. The behavior of proteins at interfaces and the mechanism of aggregation at the air-water interface have been studied extensively by many scientists and are discussed throughout this book. Generally, these studies have shown that interfacially adsorbed proteins experience a significant change of interaction forces compared to the bulk solution phase. The change in the hydrophobic/hydrophilic environment causes significant alteration of the protein's tertiary structures.

The liquid-liquid interface is present in many delivery devices, as silicone oil is commonly used as lubricant in these devices. For example, proteins are known to denature and form oil-protein particles at the silicone oil-water interface of syringes. Similarly, in delivery devices, the solid-liquid interface can promote adsorption and unfolding, where the interaction of a protein's functional groups with the solid surface determines the molecular conformation of the adsorbed state. In the

Chapter “Protein Adsorption at a Gas-Aqueous Interface”, the fundamental behavior of protein adsorption at fluid-fluid interfaces, such as the air-water interface, is discussed. The transport of proteins to the interface and the adsorption thermodynamics and kinetics are elucidated for single-component systems as well as multicomponent systems, where competitive adsorption occurs. More importantly, this understanding can define potential mitigation strategies. In the Chapter “Interfacial Behaviors of Proteins”, interfacial dynamics of the protein layer are described. Additionally, analytical techniques used in monitoring these processes will be discussed.

Drug Substance Development

Upstream process development aims to generate the target molecule for later purification and evaluation. During this process, proteins produced from bioreactors contact two interfaces: the air-water interface and liquid-solid interface (reactor walls). Although development of cell lines as well as increasing the efficiency of protein production through optimization of culture media and the manipulation of biological pathways is critical, this book will focus on the unit operations where interfacial stress impacts drug substance development the most. Thus, we will not discuss the interfaces involved in upstream process. Rather, we will focus on downstream processes where interfacial phenomena play essential roles as described below.

The purpose of downstream processing is to isolate proteins from cells or cell culture media and purify them from impurities and host cell proteins while preserving the protein’s critical quality attributes. Additionally, downstream processing prepares bulk drug substance for drug product development [9]. These processes must preserve the primary, secondary, and tertiary structures of the protein’s native state as well as minimize the formation of aggregates and particles. Downstream purification of proteins includes chromatography, filtration, and viral inactivation operations. The most common interfacial stress encountered during this stage is the stationary phase of chromatographic columns and liquid-membrane interfaces. Polymeric materials are often used as stationary phase in chromatography, where proteins are separated from impurities through adsorption at the liquid-solid interface. By manipulating polarity of the mobile phase and judiciously selecting the material surface of the stationary phase, proteins can be separated from impurities and contaminants, and, thus, they are purified. Additionally, ultrafiltration/diafiltration (UF/DF) is frequently used to exchange the chromatography mobile phase for the desired formulation buffer and for concentrating drug substance for later conversion to drug product. In the Chapter “Interfacial Stresses during Drug Substance Purification Processes”, specific interactions involved in protein purification are discussed, as well as the common practices in the industry.

Drug Product Development

Manufacturing of a biologic drug product from drug substance often involves the following steps: thawing, pooling, compounding, mixing, sterile filtration, filling, crimping, quality control, labeling, and product release. Significant efforts are made on both formulation and process development at a smaller scale prior to manufacturing the first clinical drug product batch (Fig. 2) to both inform a robust operating space and enable a stable drug product [10–12].

Product development of biologics includes interfacial phenomena that can potentially result in unwanted precipitation and diminished drug efficacy. The protein can encounter the air-water interface during multiple drug product manufacturing unit operations, such as pooling and mixing of bulk formulation solutions in the mixing tank before being filled into the intended container closure system. Surfactants are frequently added to protect the protein from the air-water interface.

Bulk protein solutions are often kept in a frozen state during transportation. However, the liquid-ice interface becomes a source of great interfacial stress during freeze-thaw processes. Furthermore, bulk protein solutions are often stored in plastic containers, where the aqueous-plastic surface (solid-liquid) interface needs to be protected to ensure no adverse effects. Moreover, processes such as lyophilization and spray drying for storage and delivery include numerous air-water or air-solid interfaces.

During formulation development, the presence of interfacial stresses is ubiquitous, ranging from pumping during vial filling to infusion in hospitals. Evaluation of interfacial stress during product development will be discussed in the Chapter "Evaluation of Interfacial Stress During Drug Product Development". After formulation development, manufacturing processes need to be scaled up. During scale-up, proteins face many interfacial stresses and shear stress in mixing tanks, and details of stress analysis of specific unit operations during scale-

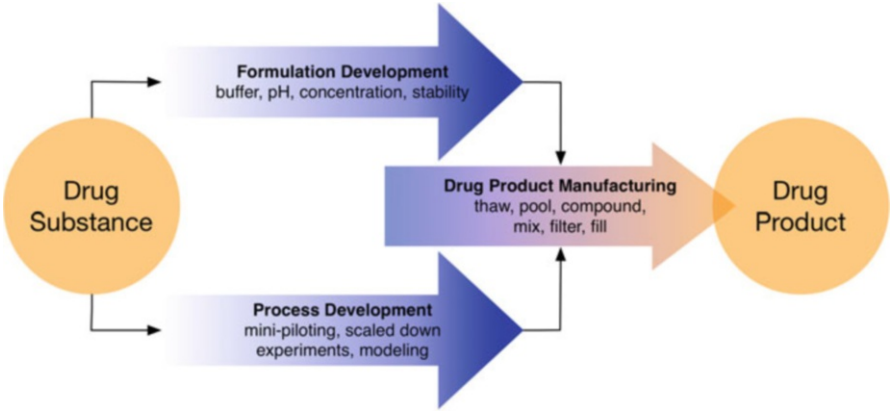

Fig. 2 Key elements of drug product development and manufacturing

up and technology transfer are described in the Chapter "Protein Interfacial Instability of Mixing and Lyophilization During Drug Product Manufacturing Process Scale-up and Tech Transfer". To suppress interfacial stress, excipients such as surfactants (polysorbates and poloxamers) are often incorporated in formulations. Various classes of excipients and their function will be described in the Chapter "Excipients: Characterization, Purpose, and Selection". While certain excipients protect proteins from interfacial stress, other excipients help to preserve proteins in their native states to maintain their therapeutic function.

Delivery systems and devices are indispensable for the administration of biologics. In the broad sense, delivery systems and devices include container closure systems, prefilled syringes, and patch pumps with delivery control. For typical container closure systems, compatibility of biologics with material surface is critical for accurate dosing. Additionally, the FDA requires testing of extractables and leachables from devices. For many functional delivery devices, such as prefilled syringes and patch pumps, the interfacial stress experienced by biologics varies from material to material. For example, for prefilled syringes, the surface energy of the tungsten wire is different from that of silicone oil (lubricant) or glass. In other delivery devices like patch pumps, proteins are exposed under pressure to the material surfaces of the cartridge and tubing. In this case, compatibility of both the chamber and tubing materials with the proteins is critical. In the Chapter "Interfaces in Protein Drug Delivery: Device Concern" compatibility of proteins with devices as well as mitigation strategies to prevent the loss of drug activity will be discussed.

Analytical Development and Assessment of Interfacial Stress in the Solid State

Throughout development of both drug substances and drug products, analytical technology plays an essential role in understanding the integrity of proteins and their degradation profiles. Unlike small molecule pharmaceuticals, proteins form aggregates, agglomerates or particles under interfacial stresses, which is detailed in the Chapter "Relating Interfacial Shear and Dilatations Stresses to Protein Aggregation in mAbs". Detection of these degradants is critical in developing mitigation strategies and ensuring product quality. In the Chapter "Analytical Techniques for Evaluating Protein Instability at Interfaces", analytical techniques are described for detecting the behavior of proteins at interfaces, including neuron scattering, quartz crystal microbalance with dissipation monitoring (QCM-D), ellipsometry, interfacial rheology, surface tensiometery, and microscopy.

Since proteins under interfacial stress can potentially form aggregates and particles, resulting in immunogenicity concerns, detection of aggregates and particles is crucial for product development. In the Chapter "Analysis of Aggregates and Particles", analytical techniques that measure aggregates and particles are reviewed, including measurement of high molecular weight species with size exclusion

chromatography (SEC), and particulate analysis using HIAC, micro-flow imaging (MFI), and other counting techniques. This chapter also discusses measurement of changes of high-order structures using circular dichroism, Fourier Transform Infrared (FTIR), and Raman spectroscopies. This chapter will focus on the practice of using these techniques in product development.

From a stability perspective, pharmaceuticals in the solid-state have an advantage because of the diminished molecular mobility. In the Chapter "Interfacial Stress and Proteins Prepared in the Solid State", interfacial stress for proteins in the solid state is discussed. Protein formulations in the solid state are typically prepared through lyophilization or spray drying. During lyophilization, interfacial stress due to formation of an ice-water interface can significantly affect protein structure and stability. During spray drying, the interfacial stress at the nozzle and at the interface of the shrinking droplet can also impact protein structure.

Concluding Remarks

The biopharmaceutical industry continues to provide healthcare benefits for patients by treating diseases such as cancers and neurological diseases through development and delivery of novel biologic drug products. New biological entities, such as RNA and DNA therapeutics, or bispecific antibodies and nanobodies, are under development and will be further explored for treating many different types of diseases. New manufacturing technologies and processes, such as disposable reactors are being implemented in many companies. Efforts around process analytical technologies (PAT) used for quality control in bioprocesses are increasing to better answer the call from regulatory agencies for enhanced process understanding and control. The implementation of novel drug-device combinations can aid in patient convenience by enabling at home or in-clinic administration, as opposed to the patient needing to receive treatment strictly in a hospital setting. Development of these new biological entities, delivery and manufacturing technologies, and analytical technologies will be discussed in the Chapter "Future Perspectives".

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Protein Adsorption at a Gas-Aqueous Interface

Ankit D. Kanthe, Raymond Tu, and Charles Maldarelli

Abbreviations

1/κ	Debye Length
BSA	Bovine serum albumin
c*	Critical bulk concentration
c_o	Bulk concentration
D	Mass diffusion coefficient
HIC	Hydrophobic-interaction chromatography
j_s	Kinetic adsorption rate
K	Energy of interaction between the adsorbed protein molecules
NMR	Nuclear magnetic resonance
Γ	Surface excess concentration
Γ*	Critical surface concentration
Γ_∞	Maximum surface concentration
Γ_{eq}	Surface equilibrium concentration
ΔG_u	Free energy of unfolding
ε_a	Activation energy for adsorption
ε_d	Activation barrier for desorption
Θ	Fractional area occupied by a molecule, Γ/Γ_∞

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μ	Chemical potential
π	Surface pressure
π^*	Critical surface pressure
γ	Surface tension
ω_p	Area per molecule at the interface
$\tau_{\text{induction}}$	Induction time for gas to liquid-expanded phase transition
ϵ	Decreased area per protein molecule due to the condensation
τ_d	Diffusive time scale
τ_k	Kinetic time scale

Introduction

Outline

The adsorption of protein molecules from a bulk aqueous phase onto fluid interfaces (an air-aqueous or oil-aqueous interface) has been the subject of decades of research. These studies derive from similar investigations into the adsorption of low molecular weight surface active molecules (surfactants) such as fatty acids and lipids, which readily adsorb to fluid interfaces. This field of study came into its own as a result of its relevance to the food industry [1–7]. Adsorption of milk and serum proteins ("hydrocolloids") to the fluid interfaces of foams and emulsion were found to both promote the formation of the dispersed phase and stabilize the dispersions from breakdown. As edible molecules, these hydrocolloids became indispensable in foam- and emulsion-based food preparations. Their biocompatibility and usefulness in preparing foams and emulsions have also made them essential in the preparation of creams and lotions for the delivery of medications to the skin and in cosmetic and hair-care products. Protein adsorption to fluid interfaces has also been studied as simplified models, in biology, for the interaction of proteins with the cell membrane and in studying physiological processes, such as lung function where the adsorption of proteins and lipids regulates the expansion and contraction of lung alveoli. The recent emergence of monoclonal antibodies as biologics for the treatment of human disease has centered attention once again on the adsorption of proteins to fluid interfaces. This adsorption occurs most importantly in the administration of these biologics from vials, syringes, or IV (intravenous) bags; the proteins adsorb to the air-aqueous solution interface at the head space [8–11] or on entrained bubbles where they can inactivate the biologic and decrease the effective dose.

This chapter outlines the basic principles in our current understanding on how proteins adsorb at interfaces and reviews the molecular structure and properties of the layers that form on adsorption. We begin in this introduction (Section "Differences Between Surfactant and Protein Adsorption to Fluid Interfaces") by describing the general characteristics of the adsorption process for proteins, contrasting this adsorption with the study of low molecular weight surfactants,

which framed the early basis of the protein investigations. Following the Introduction, Section "The Dynamics of Protein Adsorption at a Gas-Aqueous Interface" focuses on the dynamics of the adsorption process and discusses the relationship between the protein structure and the adsorption behavior. We divide this section into two parts, adsorption to a clean interface and desorption from an interface already populated with proteins. In the next section, Section "Structure and Orientation of Protein Molecules at Air-Aqueous Interfaces", the molecular structures of the adsorbed layer are described, and the details of the changing structure are reviewed. In the last section, Section "Transport Models For Adsorption of Protein to an Air-Aqueous Interface", transport models for the adsorption process are described, and simulations are compared with experimental results. Our discussion will focus principally on adsorption from aqueous solution to the air-water interface, and we will not cover adsorption onto solid surfaces, which has been thoroughly reviewed elsewhere.

Differences Between Surfactant and Protein Adsorption to Fluid Interfaces

For both surfactants and proteins, the driving force for adsorption from an aqueous solution to an air-aqueous interface is the hydrophobic effect. A protein or surfactant molecule adsorbs to the surface to remove its hydrophobic chemical moieties, which interact weakly with the large dipole moments of the water molecules through polarization forces (dipole-induced dipole). The relocation to the surface lowers the free energy as it allows water molecules to interact with each other through stronger hydrogen-bonding interactions (dipole-dipole forces). In the case of low molecular weight surfactants, the hydrophobic moieties are grouped together (the "nonpolar" part and typically in the form of methylene chains) and spatially separated from a group of hydrophilic or charged moieties ("polar" part) (Fig. 1a). The hydrophilic moieties strongly interact with water through hydrogen-bonding (dipole-dipole) or charge-dipole interactions. When these surfactants adsorb at the interface, they form a monomolecular layer with the polar group remaining in contact with the water phase to maintain hydrogen bonding with the subphase and the nonpolar group orienting either parallel or at higher surface concentrations more vertical to the interface (Fig. 1b, c).

From a mass transfer point of view, the transport of surfactant to an initially clean interface is an in-series process of molecular diffusion to the sublayer of the aqueous phase underneath the interface and kinetic adsorption from the sublayer to the interfacial zone (Fig. 1b). The step of kinetic adsorption, which initially drives the process, requires the breaking of hydrophobe/water interactions and the formation of hydrogen bonds between water molecules as the hydrophobic part of the molecule locates to the surface. As such, the process has an activation energy barrier and is described using Arrhenius rate expressions. As the surface populates with surfactant,

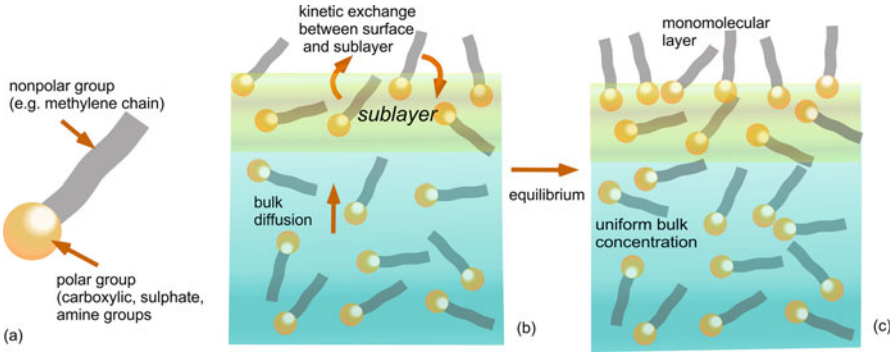

Fig. 1 (**a**) The amphiphilic structure of a low molecular weight surfactant. (**b**) A schematic illustrating the mass transfer steps in the adsorption of a surfactant from an aqueous phase to an initially clean gas-aqueous interface. Surfactant kinetically adsorbs onto the interface depleting the sublayer and driving a diffusive flux to the surface. (**c**) A monomolecular layer develops as the surface equilibrates with the bulk through adsorptive and diffusive kinetic exchange and a relaxation in the diffusion gradient

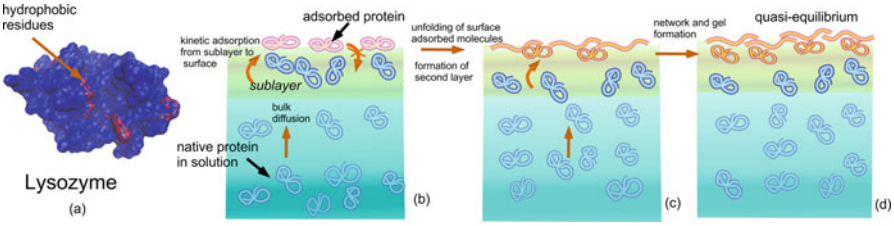

Fig. 2 (**a**) A space-filling model of the hydrocolloid protein lysozyme (PDB 3ijv), detailing the hydrophobic patches (red) of nonpolar amino acid side chains on the protein surface. (**b**) A schematic illustrating the mass transfer steps in the adsorption of protein to an initially clean gas-aqueous interface. (**c**) The unfolding of the adsorbed macromolecules, the formation of a gel-like layer, and the formation of a second layer (or multilayers) are unique characteristics of an adsorbed protein layer. (**d**) The quasi-steady adsorbed layer with the bulk concentration uniform

an equilibration follows with surfactant desorbing from the surface into the sublayer as a kinetic process also controlled by an activation energy. For adsorption to a clean interface, a true equilibrium is achieved in which the bulk concentration is uniform, and the rates of adsorption and desorption become equal, defining a monolayer surface concentration in equilibrium with the bulk phase.

In the case of proteins (Fig. 2), their molecular architecture does not typically separate into distinctly polar and nonpolar parts as with surfactants. As a result, the adsorption process itself, and the structure of the adsorbed layer that is formed and evolves, is more complex. The hydrophobic and hydrophilic moieties of the protein macromolecule are the side chains of the amino acids that extend from the backbone; alanine, valine, and leucine are examples of amino acids with hydrophobic side chains, and serine and tyrosine (uncharged) and lysine, histidine, and aspartic and glutamic acid (charged) are examples of polar side chains. In their native

configuration in an aqueous phase, proteins adopt a hierarchical structure that minimizes the free energy and provides for protein functionality (e.g., micro-environments for ligand binding and substrate binding and enzymatic reaction). This organization includes local structuring of the backbone into α-helices, β-sheets, and β-turns, which maximize hydrogen-bonding interactions of the backbone and, along with disulfide linkages between cysteine residues, provide conformational stability. This level of organization defines the secondary structure. More important from the point of view of the driving force for adsorption to an interface, the protein backbone locally folds so that the nonpolar side chains reside in the interior of a three-dimensional organization (tertiary structure) that minimizes the interaction with water molecules. In this folded state, the external surface or face of the protein's three-dimensional structure interfaces with water molecules exposing hydrophilic groups; however, some hydrophobic side chains remain on the surface (Fig. 2a). The driving force for proteins to adsorb to an air-water or oil/water interface is then the free energy gain by removing the interactions of the hydrophobic side chains on the face of the protein with water.

As with surfactants, the transport of proteins from an aqueous phase to a clean air-water interface is a process of molecular diffusion and kinetic adsorption (Fig. 2b). The kinetic step again involves an activation barrier, but the kinetic rate is slower relative to surfactants due to the fact that the protein is a macromolecule with a much larger molecular weight (10–100 kDa) and the hydrophobic surface patches are distributed around the surface. Thus, as the protein adsorbs at the surface, its reduced flexibility and size make it difficult to adopt a surface orientation that removes an optimum number of hydrophobic patches from contact with water – a task made more difficult by the fact that the patches are scattered about the surface of the protein. Thus activation energies are necessarily higher and the kinetic rates slower compared to low molecular weight surfactants.

Adsorbed proteins behave in ways that set them apart from low molecular weight surfactants (Fig. 2c, d). Molecular fluctuations of the adsorbed protein molecule can result in configurations that expose buried hydrophobic residues to the air side of the interface. These configurations are energetically more favorable than when they occur in the bulk where they simply bring the hydrophobic side chains in unfavorable contact with water molecules. As a result of the surface fluctuations, the protein can in principle follow a kinetic pathway in which these unfolding steps cascade and result in a stable "denatured" surface configuration in which the protein loses parts of the secondary and tertiary structure. Loss of native structure has three consequences. First, multilayers can form underneath the unravelled structure as hydrophobic moieties become exposed and can aggregate with hydrophobic patches of proteins that form secondary layers. Second, the unravelling can give rise to a network, which imparts to the layer large elastic and viscous moduli resisting area and shear deformations of the surface. The increase in the elastic stiffness and viscosity of the layer can be anticipated from the fact that the proteins are by themselves of large molecular size and the network formation can give rise to a pronounced gel-like behavior on the surface. Third, because of the stability of the unfolded states of the surface-adsorbed proteins, their desorption would be characterized by large

activation barriers, and, as a result, the surface-adsorbed molecules can become permanently adsorbed to the surface. The following sections will make clear these unique features of protein adsorption and how they determine the general behavior of proteins at a fluid interface.

The Dynamics of Protein Adsorption at a Gas-Aqueous Interface

In this section, we study, *as a mass transfer process*, the dynamics as protein adsorbing from a bulk aqueous phase to an initially clean air-water interface or desorbing from an already populated interface back into the bulk. As explained in Section "Differences Between Surfactant and Protein Adsorption to Fluid Interfaces", this mass transfer is an in-series process of diffusion from the bulk to the sublayer of liquid underneath the surface (and from the sublayer back into the bulk) and kinetic exchange between the sublayer and the surface; see Fig. 2 of Section "Differences Between Surfactant and Protein Adsorption to Fluid Interfaces". Experimentally, the determination of the mass of protein on the interface as a function of time has been studied by radiotracer labelling, dynamic surface tension, and ellipsometry. These methods provide time-resolved measurements with resolutions in the range of tenths of seconds to minutes, and times necessary to achieve steady or near-steady behavior in the surface adsorption can extend to hours. We first briefly review these methods for measuring the adsorption dynamics.[1]

Experimental Tools for Measuring the Dynamics of Protein Adsorption to a Fluid Interface

Radiotracer labelling (Fig. 3a) involves acetylation or reductive methylation of the amino group of the protein with a weak ^{14}C β-emitting radionucleotide and recording the radioactivity emanating from the air-water interface with time with a gas flow proportional counter. In ellipsometry (Fig. 3b), linearly polarized incident light is directed at an oblique angle to the gas/aqueous interface, and the intensity of the light specularly reflected from the interface is recorded. The incident light is elliptically

[1]Other methods which have investigated the dynamics of protein adsorption such as neutron and X-ray reflectivity have much longer resolution time scales and are more suitable for quasi-equilibrium measurements or measurements on very long time scales. Spectroscopic methods such as infrared reflection-adsorption spectroscopy and sum-frequency generation have shorter resolution time scales, but, as is also true for neutron and X-ray reflectivity, are more valuable in providing information on the molecular structure of the adsorbed layer and how this structure evolves with time. We therefore study these techniques and the insights they offer in a later section on the structure of the adsorbed protein layer.

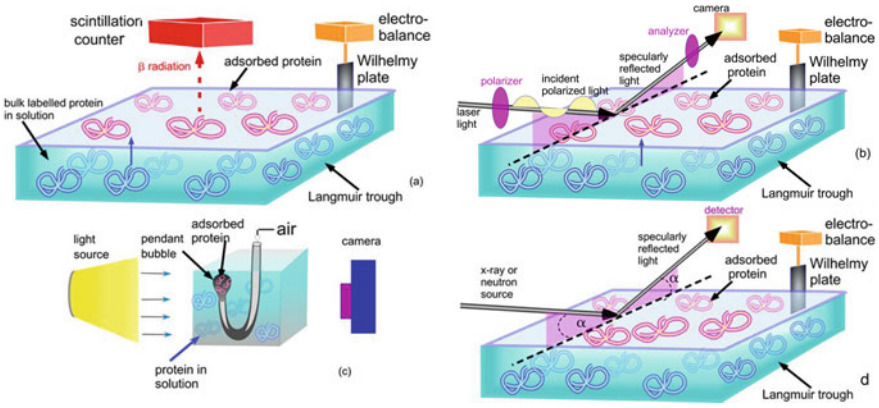

Fig. 3 The measurement of the surface concentration and thickness of protein layers on the planar interface of a Langmuir trough by (**a**) radiotracer labelling, (**b**) ellipsometry, and (**d**) X-ray and neutron reflectivity. In (**a**), (**b**), and (**d**), the dynamic tension is measured simultaneously with the surface concentration as adsorption proceeds by a Wilhelmy plate. (**c**) The measurement of dynamic tension at the surface of a pendant bubble by shape analysis

polarized by the protein surface layer, and the phase shift and tilt angle in the ellipse are measured to obtain the layer thickness and index of refraction from which the surface concentration can be obtained. In both of these techniques, the adsorption is measured on a planar air-water interface of a protein solution situated in an open (Langmuir) trough with a small depth. The interface is swept clean of protein at an initial time by aspiration to simulate conditions for adsorption to a clean interface, and the subphase underneath the surface can be exchanged with water to simulate desorption (or the protein layer can be compressed with floating booms as in a Langmuir trough arrangement).

Dynamic surface tension measurements do not directly measure the adsorbed amount but only the change in tension as adsorption proceeds. As protein adsorbs onto the interface, the surface tension (γ) is reduced from the value for a clean interface ($\gamma_o \approx 72$ mN/m at room temperature) due to the surface pressure ($\Pi = \gamma_o - \gamma$) created by the surface-adsorbed protein molecules (with concentration Γ) as described (at equilibrium) by the Gibbs-Duhem equation ($d\gamma = -\Gamma d\mu$, where Γ is the surface concentration and $d\mu = -RTd\ell nc_o$ is the change in the chemical potential of the protein in the bulk and equal to $d\ell nc_o$ for dilute solutions in which the bulk concentration c_o is changed). The changes in surface tension on adsorption were initially obtained by measuring the pull-down due to capillary forces on a Wilhelmy plate inserted into the air-water surface of a protein solution located in a trough (as with the radiotracer labelling and ellipsometry measurements); see Fig. 3a, b. However, current techniques involve forming pendant bubble from inverted needle tips immersed in the protein solution filling a transparent cuvette (Fig. 3c) or hanging drops of the solution formed from straight capillaries in air and imaging the bubble or drop shape as a function of time to measure the surface tension

(pendant bubble or hanging drop tensiometry). Other methods for the measurement of dynamic tension of proteins adsorbing to bubbles tethered to capillaries obtain the tension from the measurement of the pressure inside the bubble e.g., the maximum bubble pressure and pulsating bubble techniques.

The techniques of X-ray [12–14] and neutron reflectivity [15–17] subject the gas-aqueous interface to a highly collimated beam of neutrons or X-rays (Fig. 3d) and measure the intensity of the reflected neutrons or radiation for the specular reflection. The angle of incidence (α) is varied, and the reflectivity is collected as a function of angle (or scattering vector $q = 4\pi \sin \alpha/\lambda$, where λ is the X-ray radiation or neutron wavelength). The reflectivity is a function of the vertical density distributions in the fluid layers underneath the surface and for small angles of incidence is a strong function of the distribution in the interfacial layer of the adsorbed molecules with sub-nanometer-level resolution. Slab models of the reflectivity allow the construction of the density profiles from which the surface concentrations and thicknesses of the adsorbed layer, as well as orientations, can be obtained [18].

Using the above techniques, a number of studies on the dynamics of protein adsorption to a gas-aqueous interface have been undertaken, beginning with the pioneering studies of Davies et al. and others cited in his early review article [19], MacRitchie and Alexander [20–23], and Graham and Phillips [24–29]. Notable more recent studies are Damodaran et al. [30–36], Dickinson [4, 37, 38], Kilpatrick and Carbonell et al. [39], Andrade et al. [40], Franses et al. [41–44], and Miller, Fainerman, and Noskov et al. [3, 45–60], Wierenga, Meinders, and de Jongh [6, 61, 62] and Penfold and Thomas, and Lu et al. [15, 16]. All these investigations used primarily proteins of importance to the food industry, such as lysozyme, β-casein, bovine serum albumin, ovalbumin, and β- and γ-lactoglobulin. From these studies, a set of general characteristics pertaining to the dynamics of the adsorption process have been established, and an understanding has been obtained on the relationship between the dynamic behavior and the structural characteristics of the proteins and the physicochemical conditions of the solution (e.g., concentration, pH, and ionic strength). In the following two subsections, we discuss these characteristics providing examples from the food protein literature, for adsorption to a clean interface (Section "General Characteristics of Adsorption to a Clean Surface") and desorption from an already adsorbed layer of proteins (Section "Desorption From Already Adsorbed Protein Layers").

General Characteristics of Adsorption to a Clean Surface

We consider first adsorption of protein from a bulk aqueous phase to an initially clean air-water interface. Figure 4a details the general behavior that is observed in the surface concentration Γ as a function of time, and Fig. 4b provides the corresponding measurement of the surface tension, all for different values of the bulk concentration c_o of protein in the aqueous phase. With adsorption, the surface concentration (Γ) increases, and the tension (γ) relaxes from the clean value. The

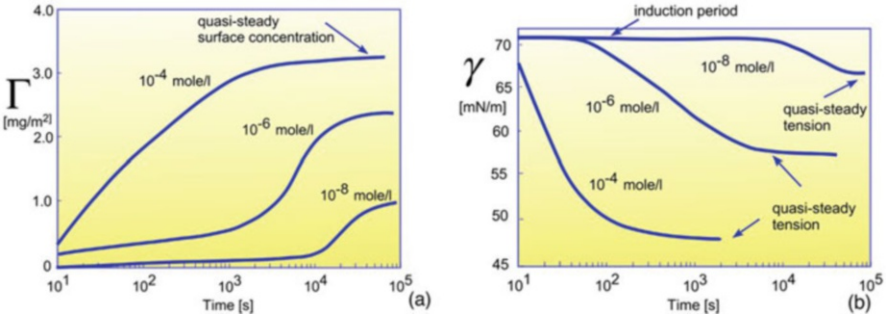

Fig. 4 General features of the dynamic adsorption of protein to a clean air-water interface with time. (**a**) Surface concentration Γ and (**b**) dynamic tension γ for different values of the bulk concentration c_o of protein in the aqueous phase

rates of these processes increase with bulk concentration c_o. At each concentration, the tension and surface concentration plateau at long times, and the times for which the plateau behavior is observed decrease with the bulk concentration. The plateau behavior is typically observed to be quasi-steady, in that the tension is observed to slowly decrease and the concentration to slowly increase. Nevertheless, from the quasi-plateau behavior, equilibrium adsorption isotherms are constructed of the quasi-equilibrium adsorption (Γ_{eq}) as a function of the bulk concentration (c_o). The schematics in Fig. 4a, b also provide typical values for the bulk concentrations of protein (10^{-8}–10^{-4} M) and typical time scales involved to achieve quasi-equilibrium, which can be upward of 10^5 s for low concentrations ($\approx 10^{-8}$M) to order 10 s at the highest concentrations ($\approx 10^{-4}$ M). The characteristic tension reductions, which vary from ≈72 mN/m to 45 mN/m (at the highest bulk concentrations, e.g., 10^{-4}M), and surface concentrations which in mass units vary from 0 to 4.0 mg/m^2 at the largest c_o are also shown.

The rate of increase in the surface concentration with the bulk concentration, as shown in Fig. 4a, can be attributed to a mass transfer effect: When a clean interface is created, protein adsorbs from the sublayer to the surface via the kinetic step, depleting the sublayer concentration. This drives a diffusion process in which protein from the bulk diffuses to the surface to replenish the sublayer. Both the kinetic and diffusion processes are accelerated with larger bulk concentrations, and this explains the increase in the rate of adsorption (and tension reduction) with bulk concentration. The generalizations shown in Fig. 4a, b are for room temperature. An increase in temperature also results in an increase in the adsorption rate, and this can also be explained through mass transfer arguments: The increase in temperature increases the diffusion coefficient of the protein in the bulk solution. An increase in temperature also increases the kinetic rate; since the kinetic step follows an activation energy process, an elevation in temperature increases the collision rate of the protein molecules with the surface, and a greater proportion of molecules have the required energy to overcome the activation barrier for adsorption.

One of the distinctive features of Fig. 4b is the presence of an *induction* period in the tension reduction for low concentrations of protein (c_o in the range 10^{-8} M–10^{-6} M). The origin of the induction period becomes clearer with the neutron [63, 64] and X-ray [65, 66] specular reflectivity measurements of the surface density and the molecular orientation of the adsorbed molecules at the surface at early times in the adsorption process. These studies show that proteins adsorb initially in a gaseous-like state in which the separation between molecules is much larger than their size. For proteins with an elongated shape, the molecules orient with their long axis parallel to the interface. As adsorption proceeds and the surface becomes more populated with protein, intermolecular interactions between the molecules drive a transition to an adsorption state of higher concentration. Fluorescence optical microscopy results [67, 68] indicate that this transition can proceed in time as a phase transition in which the surface is occupied by proteins in both a gaseous state and a state of higher density. In these experiments, an insoluble surfactant with an attached fluorescent dye is spread onto the surface of an aqueous layer in a trough; the protein solution is introduced into the layer, and the surface fluorescence is recorded. The dye fluorescence is quenched by water in the gaseous phase, but the dye is protected from quenching when the protein is located in the phase of higher concentration. The surface morphology, as demarcated by the fluorescence, changes from fluorescing islands in a dark background (nucleation of the higher density phase) to a continuous fluorescing phase with dark spots (indicating elimination of the gaseous phase) to a uniform fluorescing phase. The tension remains constant during the transition, Fig. 4b, as the increase in adsorbed amount of protein on the surface (Fig. 4a) is taken up by the formation and growth of a higher density phase. In this view, once the surface is occupied by the high density phase, further adsorption into that phase increases the surface pressure and signals the end of the induction period as a sharp drop in tension. As the bulk concentration increases, the mass transfer rate increases. As a consequence, the phase transition takes place in a shorter period of time. At relatively high concentrations, the induction behavior is not observed within the resolution of the dynamic tension measurement.

Aside from the dependence on the bulk concentration that is illustrated in Fig. 4a, b, which is a mass transfer effect, the details of the generalized curves (exact time scales and tension reductions and amounts adsorbed) for protein adsorption to a clean interface are determined principally by the following properties:

1. **Surface hydrophobicity:** The surface hydrophobicity is a measure of the area occupied by the hydrophobic side chains on the surface of the protein. The surface hydrophobicity can be characterized by measurements of the binding of long-chain hydrophobic dyes to the protein face or the retention time for elution of proteins passed through a column of hydrophobic beads (hydrophobic interaction chromatographic column (HIC)). The larger surface hydrophobicities result in greater energies gained by the relocation of the protein to the surface as the dipole-induced dipole interaction of the hydrophobic side chains with water molecules is replaced by the hydrogen-bonding interactions of these water molecules with other bulk-phase water molecules.

2. **Structural flexibility (conformational stability):** Proteins are conformationally stabilized by their secondary structures (α-helices and β-sheets), disulfide bonds, and opposite charge electrostatic interactions in the side chains of the amino acids in their tertiary structure. A measure of the flexibility of the protein (its 3D conformational stability) is its free energy of unfolding (ΔG_U) in bulk solution. This quantity is measured by using temperature or chemical denaturants to unfold the protein in solution and determining by spectroscopic methods (circular dichroism, IR, NMR, or fluorescence) the ratios of the folded to unfolded states. Proteins with larger flexibility more easily relocate onto the interface and conform to orientations that remove their surface hydrophobic patches from water. Hence the activation energy for adsorption is reduced, and a faster kinetic rate is obtained. These conformational changes on adsorption are separated from the further kinetic steps of unfolding the 3D structure to expose the hydrophobic core residues.
3. **Electrostatic interactions between adsorbed protein and protein adsorbing to the surface:** The surface charge on the protein is determined by the amino acid side chains which can become charged (i.e., amine and carboxylic acid groups). The pH of the solution determines the extent of ionization of these groups. If the protein is charged, i.e., if the pH is below or above the protein's isoelectric point (IEP), then as the protein adsorbs onto the surface, an interfacial surface charge and surface potential build up. Counterions in the bulk (typically from the suspending buffer) act to screen this charge, resulting in a diffuse layer of charge opposite in sign to the surface charge and with a thickness equal to $1/\kappa$, where κ is the inverse Debye length and is proportional to square root of the bulk concentration of the buffer counterion concentrations or the ionic strength, I. Protein diffusing to the surface through the double layer experiences the repulsion due to the surface charge, which decreases the rate of adsorption.

To illustrate how the above properties determine the dynamics of the protein adsorption to an air-aqueous interface, we describe the behavior of three hydrocolloids, lysozyme, bovine serum albumin, and β-casein, all of which have been extensively studied. Their secondary and tertiary structures are shown in Fig. 5,

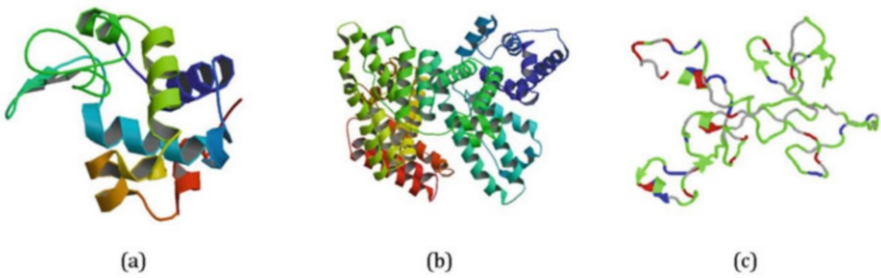

Fig. 5 The native structures of (**a**) lysozyme (PDB 2q2m) and (**b**) bovine serum albumin (PDB 1ao6) obtained from their X-ray crystallographic forms and (**c**) β-casein obtained from an energy minimization [65, 69]

Table 1 Characteristics of lysozyme, bovine serum albumin, and β-casein

Protein	Molecular weight[a] (kDa)	Isoelectric point[a]	Charge[a] at pH 7	ΔG_U[a] (kJ/mol)	HIC[a] (min)	Foam height[b] (mm)
Lysozyme (c-type)	14.6	11.35	8	35–42	18.0	0.1
Bovine serum albumin	66.4	4.7	−18	12–15	6.7	15
β-casein	23.8	5.3	−13	–	∞	16

[a]Data tabulated from Fischer et al. [70]

[b]Data from Tripp et al. [40]; HIC in phenyl sepharose hydrophobic interaction column, elution detected by adsorbance at 280 nm; foam height measured for 5 mL aliquots shaken in 20 mL vials for 30 s followed by 30 s coalescence. Protein concentrations in both tests are 1 mg/mL in pH 7.4 phosphate-buffered saline.

and their properties are detailed in Table 1. Lysozyme (specifically here chicken or c-type) is a small protein (129 amino acids, MW = 14.6 kDa) with a secondary structure 45.7% α-helices, 19.4% β-sheets, and 22.5% β-turns. Lysozyme's 3D conformation, obtained from X-ray analysis of its crystalline form, which is the most stable of the three (ΔG_U=35–42 kJ/mol, making it a hard or rigid protein), is shaped as a globular prolate ellipsoid, with dimensions equal to 45 Å × 30 Å× 30 Å. Bovine serum albumin is a much larger protein (580 amino acids, MW = 66.4 kDa), with a secondary structure 67% α-helices and 16% β-sheets and consisting of three domains. It is not as stable or rigid as lysozyme (ΔG_U=12–15 kJ/mol) and is also shaped as a globular prolate ellipsoid 116 Å × 27 Å× 27 Å. β-Casein is of intermediate size (209 amino acids, MW = 23.8 kDa) with a random coil native conformation with little tertiary structure. Importantly, the charge distribution in β-casein is nonhomogeneous, with one third of the entire charge at the *N*-terminus, making this protein, to a first approximation, similar structurally to a surfactant in its spatial segregation into hydrophobic and hydrophilic parts.

The influence of a protein's surface hydrophobicity and structural flexibility in determining its adsorption behavior is first clearly evident in the foam height measurements of the three hydrocolloids in Table 1. This data, taken from Tripp et al. [40], are the measurements of the height of foam generated by shaking an aliquot of protein solution (5 mL) in a 20 mL vial at a prescribed concentration (c_o = 1.0 mg/mL), for prescribed times of shaking (30 s) and coalescence (30 s). The experiments are all undertaken in phosphate buffer of uniform ionic strength at pH 7.4 (Table 1). Foam height is determined by two factors: (1) how fast the proteins adsorb to the clean interfaces of the bubbles created on shaking and (2) the resistivity (elasticity and viscosity) of the adsorbed layers to changes in surface area. Proteins that adsorb quickly tend to form large foam volumes under reproducible shaking because the adsorption layer builds up quickly during bubble formation creating a lower surface tension allowing smaller and more numerous bubbles to form. The adsorbed layers resist bubble coalescence, and the greater the adsorption, the more resistive the layer as coalescence involves changes in surface area. β-Caseins have

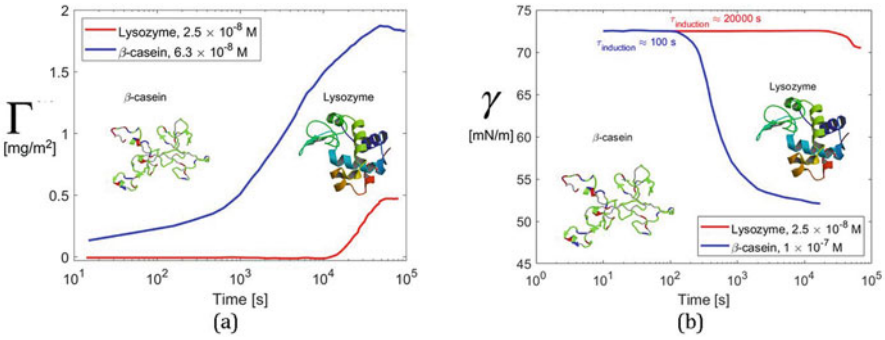

Fig. 6 (**a**) Measurement of the surface concentration of lysozyme [33] and β-casein [34] adsorbing from solution onto the planar air-aqueous interface of a Langmuir trough by radiotracer labelling. (**b**) Measurement of the dynamic tension (Wilhelmy plate) accompanying the lysozyme adsorption in (**a**) on the surface of the Langmuir trough and measurement of the dynamic tension of β-casein onto the clean interface of a pendant bubble [53] at a concentration close to the radiotracer labelling surface concentration measurements in (**a**) for this protein

the largest foam height, indicative of its flexibility as a random coil and infinite retention in its HIC measurement. In comparing bovine serum albumin with lysozyme, lysozyme has a markedly smaller foam height, with an energy of unfolding that is much larger than bovine serum albumin, while a HIC that is larger – suggesting that lack of flexibility is more important than larger surface hydrophobicity in determining the rate of adsorption.

More precise measures of the rate of adsorption of proteins from solution to a clean air-water interface are obtained by dynamic measurements of the surface concentration and the surface tension. Fig. 6a shows the measured adsorption to a clean, planar, air-water interface from a sublayer of protein solution (phosphate-buffered saline, pH 7.0, I = 0.1 M) using the radiotracer labelling technique for β-casein (c_o=6.3 × 10 $^{-8}$M) and lysozyme (c_o=2.5 × 10 $^{-8}$M) from Damodaran et al. [33, 34]. Although the β-casein concentration is 2.5 times larger (which increases the mass transfer rate), the rate of its initial adsorption is two orders of magnitude faster, indicating, as with the foam height results, the dominant importance of β-casein's structural flexibility. Lysozyme as a "hard" globular protein adsorbs slower to a clean interface. The surface concentrations plateau to quasi-equilibrium values at long times is in agreement with general characteristics of protein adsorption to a clean interface as detailed in Fig. 4a. The bulk concentrations used for these two proteins are low, and hence the times to plateau behavior are extended (order 10 ^{5} s). The dependence of the surface concentrations of lysozyme with time is particularly interesting because it indicates negligible adsorption up to 10^4 s followed by an increase in the surface concentration. An understanding of this behavior can be gained by examining the behavior of the dynamic surface tension accompanying the adsorption. The dynamic tension is obtained from Wilhelmy plate measurements that are simultaneously executed with the pendant bubble-derived surface concentration measurements (Fig. 6b) [33]. The tension relaxation for lysozyme clearly