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# Quantification of the influence of protein-protein interactions on adsorbed protein structure and bioactivity

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ABSTRACT

While protein-surface interactions have been widely studied, relatively little is understood at this time regarding how protein-surface interaction effects are influenced by protein-protein interactions and how these effects combine with the internal stability of a protein to influence its adsorbed-state structure and bioactivity. The objectives of this study were to develop a method to study these combined effects under widely varying protein-protein interaction conditions using hen egg-white lysozyme (HEWL) adsorbed on silica glass, poly(methyl methacrylate), and polyethylene as our model systems. In order to vary protein-protein interaction effects over a wide range, HEWL was first adsorbed to each surface type under widely varying protein solution concentrations for 2 h to saturate the surface, followed by immersion in pure buffer solution for 15 h to equilibrate the adsorbed protein layers in the absence of additionally adsorbing protein. Periodic measurements were made at selected time points of the areal density of the adsorbed protein layer as an indicator of the level of protein-protein interaction effects within the layer, and these values were then correlated with measurements of the adsorbed protein's secondary structure and bioactivity. The results from these studies indicate that protein-protein interaction effects help stabilize the structure of HEWL adsorbed on silica glass, have little influence on the structural behavior of HEWL on HDPE, and actually serve to destabilize HEWL's structure on PMMA. The bioactivity of HEWL on silica glass and HDPE was found to decrease in direct proportion to the degree of adsorption-induce protein unfolding. A direct correlation between bioactivity and the conformational state of adsorbed HEWL was less apparent on PMMA, thus suggesting that other factors influenced HEWL's bioactivity on this surface, such as the accessibility of HEWL's bioactive site being blocked by neighboring proteins or the surface itself. The developed methods provide an effective means to characterize the influence of protein-protein interaction effects and provide new molecular-level insights into how protein-protein interaction effects combine with protein-surface interaction and internal protein stability effects to influence the structure and bioactivity of adsorbed protein.



## 1. Introduction

The interaction of proteins with material surfaces is of primary importance in many areas of biotechnology and biomedical engineering, including biosensors, enzyme based technologies, tissue engineering and regenerative medicine, implants, and biodefense. The key element in all of these applications is the bioactive state of the protein, which can be strongly influenced by adsorption-induced changes in a protein's structure on an adsorbent surface. While much work on this topic has already been reported, a fundamental understanding on the role of different material surfaces on the conformational state, packing arrangement, and bioactivity of adsorbed proteins is still not well understood. These limitations are partly due to the complexities introduced by protein-protein interactions on the adsorption responses of proteins with various levels of internal protein stability in combination with protein-surface interactions [1–3].

As previously described by Norde [4–6] and others [7–11], when a material is exposed to a protein-containing solution, proteins rapidly adsorb to its surfaces. Once adsorbed, forces between the protein, surface, and solvent (e.g., electrostatic, hydrogen bonding, hydrophobic, and/or dispersion interactions) can alter the thermodynamic state of the system leading to spontaneous shifts in an adsorbed protein's structure from its native state and subsequent unfolding and spreading out on the surface. The amount that an adsorbed protein will unfold and spread out on a surface is largely determined by the strength of the protein-surface interactions relative to the internal stability of the protein. The extent to which unfolding will occur is also influenced by whether or not the adjacent areas of the surface are occupied by other adsorbed proteins and subsequent interactions with such neighboring proteins; which, when present, result in protein-protein interactions that

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tend to sterically block further unfolding and spreading. The degree to which protein-protein interaction effects limit the unfolding or spreading of a protein on a surface can thus be simply controlled by adjusting the concentration of the protein in solution, which influences the rate that neighboring sites are filled.

At surface saturation, the conformational state of the final resulting adsorbed layer of protein will thus be dependent on the combined influences of internal protein stability, protein-surface interaction, and protein-protein interaction effects. Protein-protein interaction effects are the least understood of these types of interactions and can be generally expected to be proportional to the amount of the protein adsorbed on the surface (i.e., areal density of the protein on the surface) [2]. Based on this assumption, the influence of protein-protein interaction effects on the structure of adsorbed protein for a given type of surface should be able to be assessed by adsorbing the protein to the surface under conditions that will provide different degrees of areal density, which can be controlled for a given surface by varying the protein solution concentration from which the protein is adsorbed, with higher solution concentrations generally resulting in higher areal densities at surface saturation [12].

The objective of this research was therefore to study the influence of protein-protein interaction effects on the structural changes and corresponding bioactivity of adsorbed protein on three different surface chemistries, each with the potential to interact with proteins through a distinctly different molecular mechanism. The experimental approach that we designed to address these issues was to first adsorb the protein from varying solution concentrations for a period of time previously determined to be sufficient to saturate the surface (adsorption time) in order to vary the initial areal density of protein on the surface and the subsequent degree of protein-protein interaction effects occurring within the adsorbed layer of protein. We then rinsed the surfaces with pure buffer to remove weakly adsorbed proteins, replaced the protein solution with pure buffer solution to remove the ability of new proteins to adsorb to the surface, and allowed the adsorbed protein layers to equilibrate under pure buffer conditions while monitoring their areal density and conformational structure by measuring the shift in absorbance and circular dichroism (CD), respectively, until they stabilized to an apparent equilibrated state (equilibration time). Following equilibration, bioactivity studies were then finally conducted to quantify the influence of the applied adsorption processes on the bioactive state of the adsorbed protein. Under these experimental conditions, differences in the optical characteristics of the adsorbed HEWL layers with different areal densities on a given surface can be considered to occur under constant internal protein stability and protein-surface interaction conditions, thus isolating the influence of protein-protein interaction effects on the structural and bioactive response of the adsorbed protein.

## 2. Experimental methods

### 2.1. Protein and material surfaces

Hen egg white lysozyme (HEWL) was selected for use in this study as one of the most well characterized protein model systems [12–19]. Being a small (MW 14 kDa) relatively 'hard' protein with 4 disulfide bonds stabilizing its structure, HEWL is generally considered to be a protein with relatively high internal stability (i.e., high internal protein stability) [2,18].

The selected material surfaces included fused silica glass (glass), high density polyethylene (HDPE), and poly(methyl methacrylate) (PMMA). These three materials were chosen to represent some of the most commonly used materials in biotechnological and biomedical engineering applications [19–25]. They were also selected because their chemical compositions provide them with the potential to interact with proteins by three distinctly different mechanisms.

Being composed of a silicon-oxygen network with a high density of hydroxyl groups on the surface, the glass surface has strong potential to form both accepting and donating-type hydrogen bonds with hydrogen bondable groups of a protein as well as ionic groups for electrostatic interactions. Because hydrogen bonds stabilize the secondary structures of a protein (as well as playing a role in tertiary structural stability), this type of surface thus has the potential to substantially destabilize a protein's secondary and tertiary structures by competing with the hydrogen bonds that serve to stabilize the protein's internal structure.

In contrast to glass, HDPE is entirely composed of saturated nonpolar alkane chains, thus lacking the ability to interact with a protein via either hydrogen bonding or electrostatic effects, while having the potential to exhibit strong hydrophobic interactions with a protein's hydrophobic amino acid residues. Given the fact that the tertiary structure of a protein is generally stabilized by hydrophobic interactions, HDPE thus has the potential to strongly induce tertiary unfolding of a protein, which in turn can be expected to potentially destabilize the native secondary structures as well.

Our third surface, PMMA, can be considered to have much lower potential to interact with the secondary structure of proteins compared to the glass surfaces since it has a much lower density of hydrogen bondable groups, with these representing only hydrogen-bond-accepting groups but not hydrogen-bond-donating groups. In addition, because the hydrogen bondable groups that are present in PMMA subsequently reduce the hydrophobicity of the surface, it can be expected to exhibit weaker hydrophobic interactions with proteins compared with HDPE. Therefore, theoretically, PMMA should exhibit lower protein-surface interaction effects than either glass or HDPE, with the greater protein-surface interaction effects from glass and HDPE occurring through distinctly different mechanisms.

### 2.2. Material surface preparation and characterization

#### 2.2.1. Preparation of material surfaces

Custom cut glass slides ($0.375'' \times 1.625'' \times 0.0625''$, Chemglass Life Sciences) were procured to fit our custom designed CD cuvettes[12]. HDPE and PMMA surfaces were spin–coated onto glass slides from dodecalin (0.5% (w/w) at 1500 rpm for 60 s) and chloroform solutions (1.5% (w/w) at 1000 rpm for 60 s), respectively. All chemicals including the HDPE ($M_w$ = 125,000, Sigma 181900) and PMMA ($M_w$ = 350,000, Sigma 445746) and the solvents such as dodecalin (Sigma 294772) and chloroform (EMD Chemicals, CX 1054) were used as supplied by the manufacturer.

Glass substrates used for adsorption studies were cleaned by sonicating in "piranha" (7:3 (v/v) $H_2SO_4$ (EMD Chemicals, SX 1244)/$H_2O_2$ (Ricca Chemicals, 3821) and basic solution (1:1:3 (v/v/v) $NH_4OH$ (BDH Chemicals, BDH3016)/$H_2O_2$/$H_2O$) at 50 °C for 1 min. Prior to adsorption studies, all the substrates were rinsed in absolute ethanol, followed by nanopure water and then dried under a steady stream of nitrogen gas.

#### 2.2.2. Characterization of material surfaces

Surface characterization was performed to determine the static air–water contact angle, atomic composition, film thickness, and surface roughness of the substrates used. For all the surfaces, the static air–water contact angle values were analyzed using a contact–angle goniometer (Kruss, DSA-20E). Similarly, the atomic compositions were verified via X–ray photoelectron spectroscopy (NESCA/BIO, University of Washington) and the average surface roughness was analyzed using atomic force microscopy (Asylum Research, MFP–3D) over an area of 5 μm × 5 μm. The thicknesses