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14. ABSTRACT The overall objective of this research was to exploit protein engineering and fluorescence single-molecule methods to enhance our understanding of the interaction of proteins and surfaces. Given this objective, the specific aims of this research were to: 1) exploit the incorporation of unnatural amino acids in proteins to introduce single-molecule probes (i.e., fluorophores for fluorescence resonance energy transfer (FRET) measurements), 2) demonstrate the utility of site-specific labeling for characterizing surface-induced conformational-bias of proteins at the single-molecule level, and 3) correlate changes in protein structure and interfacial dynamics (i.e., adsorption, diffusion,					
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Goals and Objectives

The overall objective of this research is to exploit protein engineering and fluorescence single-molecule methods to enhance our understanding of the interaction of proteins and surfaces. Given this objective, the specific aims of this research are to: 1) exploit the incorporation of unnatural amino acids in proteins to introduce single-molecule probes (*i.e.*, fluorophores for fluorescence resonance energy transfer (FRET) measurements), 2) demonstrate the utility of site-specific labeling for characterizing surface-induced conformational-bias of proteins at the single-molecule level, and 3) correlate changes in protein structure and interfacial dynamics (*i.e.*, adsorption, diffusion, and desorption) upon interaction of freely diffusing proteins with surfaces with polar, non-polar, and polymer chemistries.

Major Accomplishments

In line with the specific goals and objectives of the proposal, the major accomplishments over the course of this project included: *1) the development and application of novel high-throughput single-molecule tracking methods to characterize protein structure and dynamics in near-surface environments and 2) the development of a novel bioorthogonal approach to site-specifically immobilize enzymes on multi-functional materials via post-translational modification.* Of note, the latter achievement was part of a collaboration with Shaun Filocamo, Joshua Uzarski, and Timothy Lawton at NSRDEC, which was initiated after this project was initiated. Although not initially included in the original proposal, this work was directly complementary to the work that was originally proposed through controlling the reactivity of enzymes with surfaces. A summary of the major achievements in each aspect of this project is provided below.

Development of High-Throughput Single-Molecule Tracking Methods

We have developed an approach to monitor protein unfolding in near-surface environments using single-molecule intramolecular Förster resonance energy transfer (smFRET). This approach,

which was developed with Prof. Daniel Schwartz at CU, involves high-throughput smFRET tracking of individual protein molecules at the solution-solid interface using total internal reflection fluorescence microscopy. Using this approach, changes in conformation can be correlated with other dynamic phenomena, such as adsorption, diffusion, and desorption, by tracking $\sim 10^6$ molecules and performing large-scale multivariate computational analyses. To demonstrate this approach, structural changes in organophosphorus hydrolase (OPH) were monitored upon adsorption on fused silica surfaces. Analysis of >30,000 trajectories enabled the observation of heterogeneities in the kinetics of surface-induced OPH unfolding with unprecedented molecular detail. Although Prof. Schwartz's group has previously used single-molecule methods to investigate interfacial phenomenon, our group was the first to utilize these methods to address fundamental questions related to protein structure on surfaces. A critical component of this approach entailed the use of genetic code expansion to incorporate unnatural amino acids, which permitted the attachment of fluorophores at precise locations. By incorporating the fluorophores at precise locations, fluctuations in smFRET efficiency could be directly related to changes in protein structure at the molecular level.

Application of Single-Molecule Methods to Correlate Protein Structure and Dynamics on Surfaces

While applying smFRET, we have presented a new model for protein denaturation at solid-liquid interfaces. Our results specifically showed that protein unfolding on surfaces is mediated by surface diffusion and occurs on isolated nanoscale sites, which are rare and distinct from the majority of the surface. Accordingly, surface-mediated unfolding may be equated to a search process, which involves exploration of a surface that contains localized denaturing sites. These sites, which are virtually impossible to eliminate entirely, arise from local changes in chemical and physical properties of the surface due to spatial heterogeneity. Importantly, these heterogeneities would be invisible using conventional ensemble-averaging methods for investigating protein structure at interfaces. Additionally, because interfacial dynamics and conformation cannot be directly observed using traditional methods, the role of diffusion on unfolding would be inaccessible using such techniques. Ultimately, our findings address a fundamental question about the mechanism of protein unfolding on surfaces and, moreover, suggest a different approach to rationally tailoring surfaces for improved biocompatibility. Namely, while most strategies for reducing protein unfolding on surfaces have focused on altering the overall hydrophobicity of a surface via a uniform coating process, our results highlight the importance of reducing spatial heterogeneity. In addition to using soluble blocking agents, spatial heterogeneity may be reduced via chemically passivating anomalous sites with hydrophilic (*e.g.*, polyethylene glycol) or zwitterionic (*e.g.*, polysulfobetaine) polymers that are protein resistant. Additionally, the effects of spatial heterogeneity on unfolding may be reduced by confining protein mobility such as via introducing nanoscale features that block protein diffusion and, in turn, limit the exploration of the surface by the protein.

Additionally, using smFRET in combination with high-throughput tracking, we also investigated the use of polymer brushes to control protein-surface interactions. In this work, we showed that the interactions of proteins with polyethylene glycol (PEG) brushes are highly complex and vary as a function of grafting density. Interestingly, results of smFRET analysis found that, as grafting density increased, the rate of adsorption of the model protein fibronectin decreased; however, adsorbed molecules unfolded faster, and unfolded molecules were retained longer on the brush. The stabilization of unfolded molecules was attributed to a reduction in hydration in regions of the

brush due to changes in the brush structure, which favor binding of unfolded proteins at these sites. These findings suggest there is an inherent balance between protein adsorption and the stabilization of unfolded protein molecules on the surface that arises from increasing grafting density. This understanding is critical for designing polymer brushes that resist unfolded protein molecules and, in turn, prevent adverse biological responses.

To test the hypothesis that the stabilization of unfolded fibronectin was due to a reduction in hydration in regions of the brush, we investigated the impact of adding the zwitterionic polymer poly(sulfobetaine) (PSB) to PEG brushes. We reasoned that the addition of PSB, which is highly hydrophilic, would disrupt local hydrophobic “hotspots” within the PEG brush, thereby reducing the stabilization of unfolded fibronectin. Our results indicated that, as the fraction of PSB in the brush increased, the rate of fibronectin adsorption decreased. However, interestingly, an optimum fraction of PSB for inhibiting the stabilization of unfolded fibronectin as well as minimizing the denaturation of fibronectin was observed. Additionally, in single-molecule experiments using an environmentally-sensitive fluorophore, we directly showed that the relative fraction of hydrophobic “hotspots” decreased with increased PSB. These results suggest that the optimum ratio of PSB-to-PEG arises from the balance between the density of hydrophobic “hotspots” and electrostatic interactions at high PSB fractions. The enhancement of electrostatic interactions between fibronectin and the brush at high PSB fractions may also be denaturing. We are currently exploring the molecular basis for the impact of mixing PSB with PEG to prevent denaturation and the stabilization of unfolded fibronectin via molecular dynamics simulations. Ultimately, these findings implicate the potential utility of mixed PEG/PSB brushes as a novel coating for biomaterials with improved biocompatibility over conventional polymer brushes.

Lastly, we have combined two-color *intramolecular* smFRET with *intermolecular* smFRET to connect surface-adsorbed protein structure and function. This approach was demonstrated by simultaneously measuring binding of the integrin $\alpha_v\beta_3$ from solution to surface-adsorbed fibronectin as a function of fibronectin conformation on the surface. Using this approach, we showed that the interaction between surface-adsorbed and $\alpha_v\beta_3$ is strongly dependent on interfacial dynamics, and that, more specifically, such dynamics directly influence both the stability of the bound fibronectin- $\alpha_v\beta_3$ complex and time-to-binding for $\alpha_v\beta_3$ to fibronectin. In particular, as the dynamic behavior of fibronectin increased, the rate of binding, particularly to folded fibronectin, and stability of the bound fibronectin- $\alpha_v\beta_3$ complex decreased significantly. Changes in dynamics may alter the rate of binding and stability of the bound complex by perturbing the local structure, accessibility, and orientation of the integrin binding site. These findings ultimately have important implications in controlling the response of cells to natural as well as foreign materials and, moreover, the underlying mechanism of the sensing of mechanical forces by cells, which are regulated by fibronectin-integrin interactions.

Development of Post-Translational Ligation Approach to Site-Specifically Immobilize Enzymes

In addition to understanding the interaction of proteins and enzymes with surfaces, we were inherently interested in also controlling the reaction of proteins and enzymes with materials. To this end, we also developed a novel bioorthogonal approach to site-specifically immobilize enzymes on multi-functional surfaces via post-translational modification. This approach, which was initially demonstrated with green fluorescent protein and later with the model enzyme lipase, was based on the modification of an engineered peptide tag by the enzyme lipoic acid ligase

(LplA). In this approach, LplA is used to modify an engineered peptide tag, which is cloned into the target enzyme, with an azide-containing substrate (*i.e.*, 10-azidodecanoic acid). Following the ligase-mediated reaction, the azide group may subsequently be used to “click” the target protein or enzyme to the surface in a bioorthogonal manner. Similar enzyme-mediated approaches to conjugate proteins with modifying agents and to materials have been reporting using other enzymes, including biotin ligase, sortase, transglutaminase, farnesyltransferase, and formylglycine generating enzyme. However, such approaches are generally restricted in terms of the location of the modification and, in some cases, non-specific while also resulting in cleavage of the polypeptide backbone of the target protein. Additionally, the substrates for these other enzymes, which are also slow and inefficient, are not readily available or easily synthesized, thereby limiting widespread use of these approaches.

Having successfully demonstrated this approach, a fundamentally important question is to what extent can we predict sites in enzymes that can accommodate the LplA tag. This question is particularly important from a practical perspective given that the insertion of large peptide loop can disrupt protein or enzyme structure and lead to denaturation. While the likelihood of accommodation of such loops may be predicted from structural analysis, the accuracy of such predictions is frequently low, which, in turn, translates to the need to experimentally test a large number of sites. To overcome this challenge, we demonstrated a computationally inexpensive method to scan protein or enzyme structures to rapidly and accurately identify sites that accommodate peptide insertions. The basis for this method is the use of the kinematic closure loop modeling feature within the Rosetta protein modeling software suite, which was used to model the LplA tag within lipase. Having modeled the LplA tag at every amino acid position in lipase, the model predictions were compared to the production of soluble enzyme for a library of lipase constructs that were created with the LplA tag at varying locations. In this case, the production of soluble lipase was used as an experimental measure of the ability of a site to accommodate the insertion. Interestingly, our results found a strongly correlation between the Rosetta score and soluble expression and, more specifically, that the prediction was dominated by the nature of the neighboring residues around the mutation site. Perhaps even more interesting, using this approach, we were able to accurately predict sites that accommodate the LplA tag that would be unintuitive based on structure and related measures, including solvent accessibility and temperature factor, alone. The ability to predict such sites *a priori* presents considerable opportunities to significantly reduce the number of sites that must be tested experimentally while facilitating the ease with which an enzyme may be immobilized on a surface in a site-specific manner.

Publications Supported by this Award

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6. Faulón Marruecos D, Kim HH, Shirts MR, Schwartz DK, Kaar JL., Controlling local hydrophobicity in poly(ethylene glycol) brushes with poly(sulfobetaine) to mediate the stabilization of unfolded fibronectin on biomaterials. (In Preparation)