The effects of tether placement on antibody stability on surfaces

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(Received 23 January 2017; accepted 4 May 2017; published online 1 June 2017)

Despite their potential benefits, antibody microarrays have fallen short of performing reliably and have not found widespread use outside of the research setting. Experimental techniques have been unable to determine what is occurring on the surface of an atomic level, so molecular simulation has emerged as the primary method of investigating protein/surface interactions. Simulations of small proteins have indicated that the stability of the protein is a function of the residue on the protein where a tether is placed. The purpose of this research is to see whether these findings also apply to antibodies, with their greater size and complexity. To determine this, 24 tethering locations were selected on the antibody Protein Data Bank (PDB) ID: 1IGT. Replica exchange simulations were run on two different surfaces, one hydrophobic and one hydrophilic, to determine the degree to which these tethering sites stabilize or destabilize the antibody. Results showed that antibodies tethered to hydrophobic surfaces were in general less stable than antibodies tethered to hydrophilic surfaces. Moreover, the stability of the antibody was a function of the tether location on hydrophobic surfaces but not hydrophilic surfaces. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4983705>]

I. INTRODUCTION

Antibodies, with their ability to selectively bind specific proteins or small molecules, have the potential to offer new or improved insights in many fields including defense,¹ medicine,² proteomics,³ sensors,⁴ biocatalysis,⁵ and bioanalytics.⁶ Many technologies currently in existence make use of the binding property of antibodies, but these usually require special equipment or highly trained users. Current efforts seek to make a device that is more portable, robust, automatic, and simple by attaching the antibody to a solid substrate to create a chip-like diagnostic.^{7,8} Prominent among such technologies are antibody microarrays where antibodies are attached to a surface to detect the presence or concentration levels of proteins of interest present in a sample. These microarrays are currently used in biomedical research.^{9–11} However, this technology has potential beyond the research environment as sensitive and rapid detectors of CBRNE (chemical, biological, radiological, nuclear, and explosive), epidemiological threats, and individual biological susceptibility.

Despite their potential benefits, current antibody microarrays suffer from poor performance. Specifically, results from current antibody microarrays are notoriously too variable to yield conclusive results in many instances.^{7,12–14} On some microarrays, even the *same* antibodies arrayed at different locations on the *same* chip have been shown to vary in activity by up to 43%.¹⁵ Since the microarray's performance is dependent on the activity of the antibody, building a reliable microarray would benefit from the knowledge of the behavior of the antibody on the surface and how the surface affects its structure. This knowledge is key to improving the design and manufacture of next generation devices.

One of the difficulties in examining the structure of an antibody comprising a microarray is that no experimental method exists to examine the protein/surface system at atomic-level resolution. Typical methods such as NMR and X-ray crystallography cannot be used with a surface present. Other methods such as TOF-SIMS (Time-of-Flight Secondary Ion Mass Spectrometry) and FTIR (Fourier transform infrared spectroscopy) can provide information about the aggregate protein structure on the surface but still lack the resolution necessary to study the protein structure on an atomic level.^{16,17} Recent advancements in atomic force microscopy (AFM) and CD (circular dichroism) spectropolarimetry combined with AAL/MS (amino acid labeling/mass spectrometry) have been able to provide improved insights into the tertiary and secondary structure of an adsorbed protein, but they still fall short of providing atomic resolution.^{18,19}

Because of the difficulty in observing proteins experimentally, molecular simulation has emerged as the primary method to study protein/surface interactions. For example, Latour and co-workers have used atomistic models²⁰ to study model peptides²¹ and proteins such as fibrinogen²² on various surfaces. Kubiak and Mulheran used atomistic models to study interactions between hen egg white lysozyme and charged surfaces.²³ The results showed adsorption trends in good agreement with the experimental work and gave more insights into the adsorption process. Raffaini and Ganazoli used atomistic simulations to model the adsorption of human lysozyme on graphite and were able to determine the final adsorption geometry of the protein.²⁴ Finally, Hung *et al.* used atomistic simulations to study the mechanism that cardiotoxin uses to adsorb onto a self-assembled monolayer.²⁵

In addition to atomistic representations, many researchers have used coarse grain models to study protein/surface interactions. Pandey *et al.* started with an atomistic model

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to determine the adsorption energy of each naturally occurring amino acid with graphene but then used a coarse grain model to study the interaction of two peptide chains with graphene.²⁶ Yu *et al.* used a coarse grain model to examine protein/surface systems at microsecond time scales²⁷ as have others due to the computational efficiency offered by such models.^{28–32} Zhuang *et al.* studied the effect of tethering on peptide folding mechanisms,³³ and coarse grain models have also been used to determine the thermodynamic properties of tethered proteins compared to their bulk counterparts.^{34–39}

Another advantage of coarse grain simulations is that they allow the thermodynamically rigorous quantification of protein stability. Determining protein stability on the surface is important because it is related to protein function,^{35–39} but in order to quantify protein stability, the free energy of folding must be calculated. Doing this in simulation requires the protein to fold and unfold multiple times. As such, an atomistic model is impractical as it would take too long to achieve statistically meaningful results. Coarse grain models, however, can sufficiently sample the relevant regions of phase space in a computationally efficient manner.

When a protein such as an antibody is tethered to a surface, it typically becomes less stable,^{40,41} which is reflected by a decrease in the melting temperature and free energy of folding compared to the bulk case. When optimizing microarrays, the question then becomes how to minimize the instability. Previous work has demonstrated that protein stability on a surface is a function of the residue where the tether is located for a small protein, and that optimal tether locations can be predicted for such molecules.³⁷ However, antibodies are much larger than the proteins studied previously. The increased flexibility and multiple domains, compared to the previously studied proteins, call into question the validity of applying the heuristics developed for small proteins to larger systems. The purposes of this study is to examine how the placement of the tether affects the stability of surface antibodies and determines which sites are optimal in maintaining the stability of the molecule as a whole and the antigen binding domain specifically.

This work also expands beyond previous efforts in other ways. Specifically, prior work was restricted to only two different orientations (upright and flat)³⁹ and did not give a complete picture of the optimal way to tether antibodies to surfaces. As such, the work presented here will examine many tethering orientations to determine the effect the tethering location has on antibody stability. The specific hypothesis tested is that tethering the antibody at a residue far from the antigen binding domain will yield more stable active sites than when tethers are placed closer. Previous work with small proteins has shown that the structure immediately around the tether location can become corrupted.^{33,36–38} It is therefore supposed that the farther the tether is placed from the active region, the more stable the active region will be.

The remainder of this paper is structured as follows. First, in Sec. II the protein and models are described in more detail. This is followed by a description of the experimental design and simulation and analysis protocols. The results are presented in Sec. III and Sec. IV contains a

discussion of their impact. Taken as a whole, this research shows that antibody stability is a function of location on hydrophobic surfaces but not on hydrophilic surfaces—a finding important to building better arrays which was not known previously.

II. METHODS

A. Protein and model

The antibody studied here is the IgG contained in Protein Data Bank (PDB) ID: 1IGT. This antibody was chosen because its structure is understood well.⁴² A ribbon depiction of the antibody is shown in panel (a) of Fig. 1, and the relevant regions of the molecule are labeled. The antibody is composed of one Fc section and two Fab sections. The Fc section can be further broken down into two regions, the C_H3 region and the C_H2 region, shown in blue and green, respectively. The Fab section may also be broken down into smaller regions—the C_L, V_L, C_H1, and V_H regions. The Fc and Fab sections are connected by a *hinge*, shown in yellow.

FIG. 1. (a) Ribbon depiction of PDB ID: 1IGT with labels of the various regions. (b) A coarse-grain model of the antibody.

The VL and VH regions comprise the active site of the antibody, the *antigen binding domain* (ABD), which is shown in red. This is the portion of the molecule that recognizes and binds with antigens and two ABDs (designated ABD 1 and ABD 2 below) that are found on each antibody. The other antibody domains are involved primarily with signaling an immune response in the body and do not participate in binding to the antigen. In fact, multiple researchers have shown that functional antibody arrays may be fabricated by placing only the Fabs on the surface rather than the entire antibody.^{43–46} Based on these results, the assumption made in this work is that the binding function of the antibody is determined by the degree to which the ABD remains properly folded regardless of the amount of unfolding that may be present in the other portions of the molecule.

Because of its size and complexity, the antibody is modeled using a coarse-grain, Gō-like model developed by Karanicolas and Brooks.^{47,48} This model treats each residue in the protein as a single interaction site placed at the C $_{\alpha}$ position of each amino acid. The secondary and tertiary structures of the molecule are stabilized by defining the so-called “native contacts.” Two residues whose heavy atoms are within 4.5 Å of each other or which form hydrogen bonds in the native, folded state are designated “native contacts” and are assigned a favorable interaction energy of the form

$$V_{ij} = \epsilon_{ij} \left[13 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - 18 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{10} + 4 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right]. \quad (1)$$

Here r_{ij} refers to the instantaneous distance between residues i and j , σ_{ij} is the distance between the C $_{\alpha}$ carbons of residues i and j in the native structure, and ϵ_{ij} is the strength of interaction between residues i and j which is based on the pair interaction energies reported by Miyazawa and Jernigan.⁴⁹ The “10” term accounts for the “desolvation penalty” in the native contact potential. The model is transferable to peptides of any type and has been used to accurately model proteins of many sizes including enzymes and antibodies.^{36,38,39,50} The model was parameterized with data at typical biological conditions (e.g., $6 \lesssim \text{pH} \lesssim 8$, $[\text{Na}] \lesssim 200 \text{ mM}$) and is not valid far away from such. However, such limitation is of little importance to this work as antibody arrays are used at biological conditions. Further details of the model can be found in the original papers.^{47,48,51,52}

Panel (b) of Fig. 1 shows the coarse grain model of the antibody. The different regions of the molecule are color-coded as in panel (a). This representation of the molecule contains 1316 sites placed at the C- α position of the 1316 residues of the molecule in accordance with the protocols outlined by Karanicolas and Brooks.

B. Surface model

The surface was modeled with the formalism described by Wei and Knotts.⁵³ This model treats the surface as a flat plane but independently accounts for the chemistry of each residue and the chemistry of the surface. The surface may vary from hydrophobic to hydrophilic, and the nature of each residue-surface interaction depends on the hydrophobicity of the residue compared to the surface. This model was developed specifically to work with the coarse-grain protein

model of Karanicolas and Brooks, and the residue-surface potential (U_{surface}) is of the form

$$U_{\text{surface}} = \sum_i^N \left\{ \pi \rho \sigma_i^3 \epsilon_i \left[\theta_1 \left(\frac{\sigma_i}{z_{is}} \right)^9 - \theta_2 \left(\frac{\sigma_i}{z_{is}} \right)^7 + \theta_3 \left(\frac{\sigma_i}{z_{is}} \right)^3 - (\theta_s \chi_s + \theta_p \chi_{pi}) \left(\frac{\sigma_i}{z_{is}} \right)^3 \right] \right\}. \quad (2)$$

Here, the summation is over all residues (N), z_{is} is the distance between the residue i and the surface, $\rho = 1.0 \text{ Å}^{-3}$, σ_i is a residue-specific van der Waals parameter, and ϵ_i is a residue-specific parameter controlling the overall strength of the interaction. The θ 's are tuning parameters with values $\theta_1 = 0.2340$, $\theta_2 = 0.4936$, $\theta_3 = 0.1333$, $\theta_s = 0.0067$, and $\theta_p = 0.0333$. The χ 's describe the chemical nature of the residues and the surface. The values of χ for both the surface and the residues are found in Ref. 53. This model has shown to be in both quantitative and qualitative agreement for adsorption experiments done for several proteins.⁵³

C. General experimental design

Testing the hypothesis was done by determining how the surface affected the stability of the protein compared to the bulk case. Specifically, the bulk case served as the control, and the treatments were all cases where the protein was tethered to a surface. A treatment consisted of a *tether location* and a *surface type*. Two types of surfaces were investigated, hydrophilic and hydrophobic, and the antibody was tethered to the surface in 24 different locations. For example, tethering to a residue ASN385 on a hydrophobic surface was one treatment and tethering to a residue GLY219 on a hydrophilic surface was another treatment. Thus, a total of 48 treatments were tested and compared to the control.

The tether location (the residue on the antibody where the tether was placed) of each treatment was chosen based on the previous work which showed that loop regions, in general, are better candidates for tethering than other options.³⁷ Most loop regions in an antibody occur between stretches of amino acids forming beta sheets, with some loops being found on the outside of the molecule and others on the inside. The interior loops were not tested because they have been shown to destabilize proteins on surfaces.

Figure 2 shows the loop region where tethers were placed in the CH3 region of the antibody. Tethering sites were also

FIG. 2. Tethering locations on the loop regions in the CH3 region of the antibody.

chosen in the CH2 and hinge regions, but these are not shown in Fig. 2 for brevity. The exact residues used from the CH3 region were ARG444, LEU433, ASN422, GLY403, LEU390, ASN385, PRO375, THR360, and ALA346. The residues from the CH2 region were SER343, PRO330, GLY317, GLN310, ASN298, HSD286, ASN282, PRO272, ASP270, and SER255. The residues from the hinge region were LEU236, LYS229, PRO226, ILE222, and GLY219.

D. Simulation and analysis protocols

All simulations were performed with in-house code. To ensure adequate sampling of the folded and unfolded states of the protein, the replica exchange method was used with 80 boxes ranging from 242 K to 400 K in 2 K increments. The Nosé-Hoover chain formalism was used to generate the canonical ensembles required for the method^{54–56} with a time step was 3 fs.

Attaching the molecule to the surface was done by placing a harmonic restraint on the residue to be tethered and holding its position 5.8 Å above the surface as has been done previously.^{37–39} The specific form of the potential was

$$U_{\text{tether}} = \frac{1}{2}k(r - r_0)^2, \quad (3)$$

where $k = 100$ kcal/mol, r is the instantaneous position of the tethered site, and r_0 is (0.0, 0.0, 5.8) Å. Previous work has demonstrated that neither the length of the tether nor the presence of an actual tethering molecule has a significant impact on the stability of the antibody because of the large size and flexibility of the molecule.⁵⁷

The initial configuration for each treatment was obtained by placing the crystal structure of the molecule above the surface and performing a brief simulation to allow U_{tether} to “pull” the tethered residue to the desired position above the surface. Each simulation then consisted of 10^7 steps of equilibration, during which data were not accumulated, followed by 3×10^7 steps of production. Proper equilibration was determined by monitoring the potential energy of the system and ensuring that it had reached equilibrium. This usually occurred in 10^5 iterations which corresponded to 0.3 ns. As has been done before, periodic boundary conditions were used but the box length was set such that the molecule could not interact with its periodic image.^{35–39} The data were analyzed using the PyMBAR analysis tool.⁵⁸

Two key parameters were used to determine how the stability of the molecule was affected by the surface compared to the control. The first is the heat capacity. During unfolding transitions, the heat capacity of a molecule peaks, and the temperature at which this peak occurs is the so-called transition temperature for the portion of the molecule that is unfolding. Transition temperature is directly related to stability and serves as a convenient metric for comparison between the control and the treatments. If the transition temperature is higher for the treatment than for the control, the surface has stabilized the protein for that tethering configuration compared to the bulk case. If the transition temperature is lower for a treatment than the control, the protein has been destabilized by the surface.

The heat capacity was calculated using the outputs from the PyMBAR analysis. PyMBAR provides the density of states, $\Omega(U)$, from which any temperature-dependent quantity of interest may be calculated using

$$X(T) = \langle X \rangle_T = \frac{\sum_U X(U)\Omega(U)e^{-\beta U}}{\sum_U \Omega(U)e^{-\beta U}}. \quad (4)$$

Here, the brackets denote the ensemble average and U is the potential energy of the system. Using Eq. (4) to calculate the needed ensemble averages, the heat capacity is found from

$$C(T) = \frac{\langle U^2 \rangle_T - \langle U \rangle_T^2}{RT^2}, \quad (5)$$

where R is the gas constant and T is the temperature. The second useful metric for describing antibody stability is the *fractional nativeness* or *fraction folded* q . Fractional nativeness is a measurement of how much of the antibody is in its native confirmation. It is defined as the number of native contacts at any given time divided by the total number of possible native contacts. A native contact occurs when two residues are within 1.2 times the distance of the residues when the antibody is in its folded state, as defined by Karanicolas and Brooks.^{47,48} A domain is considered melted when its q drops below 0.5, meaning it is more than 50% unfolded, as has been done previously.^{35–38}

The melting point most closely connected to antibody activity is the variable region on the Fab, which is denoted by VL and VH on panel (a) of Fig. 1. As described above, this region is known as the antigen binding domain (ABD) and is the location on the antibody that binds to the antigen. Much of the analysis that follows is thus focused on this particular transition temperature.

E. Statistics

Because of the large number of tethering sites, replicates could not be run for every site. It was therefore decided to run replicates on just the most stable cases. On the hydrophobic surface, three replicates were run on seven sites: ASN385, THR360, GLY317, GLN310, PRO272, PRO226, and ILE222. On the hydrophilic surface, replicates were run on six sites: PRO375, SER343, PRO330, HSD286, ASN282, and GLY219. The uncertainty was calculated from $\frac{\sigma}{\sqrt{N-1}}$, where σ is the standard deviation from N replicates.

III. RESULTS

A. Bulk stability (the control)

Figure 3 shows the heat capacity of the antibody in the bulk as a function of temperature. Notice that three peaks are present which indicate a folding mechanism with four stable states. The folded state is found at the lowest temperatures and the random coil state at the highest temperature. Two partially folded intermediates occur at intermediate temperatures. Figure 4 shows representative snapshots of the antibody in each of the stable states and are labeled consistent with those in Fig. 3. At low temperatures, the antibody is in its folded conformation (i). As the temperature increases, the CH2 domain begins to denature (ii), followed by the CH1 and CL regions

FIG. 3. Heat capacity of the antibody as a function of temperature in the bulk solution.

in (iii). Finally, the CH3 domain and ABDs denature in (iv). The CH2 domain has been shown experimentally to be the weakest domain and the fact that it denatures first is consistent with these findings.⁵⁹

The melting point of the ABDs is found from the q value for those sections of the antibody. Figure 5 shows the average q value for ABDs for the bulk case. Each line represents one of the antibody's ABDs. The shape of the curve suggests that the ABDs unfold through a similar three-step process, but they do not melt at exactly the same temperature. The melting points of the ABDs were found to be 361 K and 366 K, respectively. This difference in melting temperature is expected due to the antibody's structure. The molecule, though Y-shaped, is not symmetric and the different ABDs interact with the remainder of the molecule differently.

B. Hydrophobic surface

The folding mechanism changes when the antibody is tethered to a hydrophobic surface. For every tethering

FIG. 4. Representative snapshots of the stable states on the folding pathway of IIGT in the bulk solution.

FIG. 5. Fraction folded of the two ABDs of the antibody in the bulk.

location, the presence of the surface causes the CH2 region to denature and collapse onto the surface even at low temperatures. Thus, the most-stable state on the surface was one where the CH2 domain was denatured but the remainder of the molecule was stable. The remainder of the folding mechanism differed among the different tether locations, but they generally followed one of the three courses as is now described. Figures 6 and 7 show representative examples of the three types of folding mechanisms seen for the treatments. In panel

FIG. 6. Heat capacity curves of the antibody denaturing on a hydrophobic surface for (a) PRO226, (b) GLY403, and (c) LEU390.

FIG. 7. Representative snapshots of the antibody denaturing on a hydrophobic surface for (a) PRO226, (b) GLY403, and (c) LEU390.

(a), the entire antibody unfolds at the same time as indicated by the single peak on the heat capacity curve. Once unfolded, the random coil is absorbed onto the surface.

The second class of transitions is depicted in panel (b). Here the antibody undergoes two transitions as seen in the two peaks on the heat capacity curve and thus has three stable states.

TABLE I. Melting temperatures for the antigen binding domains on a hydrophobic surface. Average uncertainty based on replicates is ± 1.1 K.

Residue	ABD 1 temp (K)	ABD 2 temp (K)
ARG444	347	350
LEU433	347	351
ASN422	347	349
GLY403	344	354
LEU390	347	351
ASN385	348	349
PRO375	341	349
THR360	349	349
ALA346	344	353
SER343	343	352
PRO330	339	351
GLY317	347	353
GLN310	350	352
ASN298	342	349
HSD286	348	349
ASN282	346	351
PRO272	333	352
ASP270	343	346
SER255	349	348
LEU236	342	350
LYS229	342	351
PRO226	352	353
ILE222	353	346
GLY219	349	345

FIG. 8. Comparison of a more stable tethering location (PRO226) with a less stable tethering location (ASP270) on a hydrophobic surface.

The lowest-temperature, stable state is not fully folded. Rather, the majority of the antibody collapses onto the surface, but the ABDs remain intact. During the lowest temperature transition, the CH1 and CL regions of the Fab denature and collapse onto the surface, but the ABDs (the VL and VH regions of the Fab) largely remain folded. At higher temperature, the ABD itself melts and the entire molecule collapses down onto the surface.

FIG. 9. Heat capacity curves of the antibody denaturing on a hydrophilic surface for (a) ASN422, (b) ASN385, and (c) PRO272.

Panel (c) shows the third type of the transition exhibited by the treatments. In this case, the antibody undergoes several transitions involving small portions of the molecule which result in a fairly gradual, overall transition. The analysis of the trajectories indicates that four different states can be identified. At low temperatures, the CL and CH1 regions denature and collapse onto the surface. Subsequent melting occurs first in the ABD region. The last region to melt in this case is the CH3 region.

As indicated previously, the stability of the antigen binding domain is the most important in terms of antibody function. The transition temperatures of the ABD for the 24 treatments on the hydrophobic surface are shown in Table I. In general, the tethering locations with higher transition temperatures occur for cases that only underwent one or two transitions [cases (a) and (b) of Fig. 6]. Most of the treatments that resulted in less stable ABDs underwent three or more transitions [panel (c) of Fig. 6]. Interestingly, the destabilization of the ABD was accompanied by a stabilization of the CH3 region as indicated in panel (c) of Fig. 6.

As seen in Table I, the highest ABD transition temperature occurs on ABD2 when tethering at GLY403 and is 354 K. Recall that the ATB transition temperatures in the bulk were 361 K and 366 K for ABD 1 and 2, respectively. It therefore appears that hydrophobic surfaces always destabilize the ATB relative to the bulk case. However, the degree to which

it destabilizes is a function of the tethering location. In the least stable case, the ABD began melting at 333 K. Thus, the difference between the most stable and least stable treatments on the hydrophobic surface is more than 20 K. Figure 8 shows a comparison of a less stable case (ASP270) with a more stable case (PRO226). Both of these cases undergo a single transition, but when the antibody is tethered at PRO226, the peak occurs 15 K higher than when it is tethered at ASP270.

C. Hydrophilic surface

As with the hydrophobic surface, when the antibody is tethered to a hydrophilic surface, it undergoes several different folding mechanisms. Figures 9 and 10 show representative snapshots and heat capacity curves for three of these folding mechanisms. In panel (a), the majority of the antibody denatures in the first transition while the ABDs denature during the second transition. The folding mechanism in panel (b) is similar except that the CH3 region denatures in a separate transition after the ABDs. In panel (c), the folding mechanism is identical to panel (b) except that the ABDs denature after the CH3 region. There were also several cases where the unfolding occurred in an uncooperative way such that the transition was very broad and no specific domain dominated at any particular temperature.

The heat capacity curves for the hydrophilic cases generally had multiple, sharp peaks similar to the bulk case. The actual number of peaks varied from case to case, but for many locations, the antibody underwent the same number of transitions as in the bulk. Although some domains increased in stability, such as the CH3 region in panel (c) of Fig. 9, the

TABLE II. Melting temperatures for the antigen binding domains on a hydrophilic surface. The average uncertainty based on replicates is ± 1.4 K.

Residue	ABD 1 temp (K)	ABD 2 temp (K)
ARG444	359	365
LEU433	363	365
ASN422	362	365
GLY403	363	363
LEU390	360	363
ASN385	359	368
PRO375	363	367
THR360	360	367
ALA346	360	364
SER343	361	367
PRO330	361	368
GLY317	359	367
GLN310	358	364
ASN298	359	363
HSD286	362	367
ASN282	362	368
PRO272	360	368
ASP270	360	368
SER255	361	366
LEU236	359	368
LYS229	360	368
PRO226	362	365
ILE222	363	365
GLY219	363	366

FIG. 10. Representative snapshots of the antibody denaturing on a hydrophilic surface for (a) ASN422, (b) ASN385, and (c) PRO272.

FIG. 11. Comparison of a more stable tethering location (PRO375) with a less stable tethering location (GLN310) on a hydrophilic surface.

ABDs' stability on the surface remained largely unchanged compared to the bulk behavior.

Table II shows the melting point of the ABDs for each of the tethering locations on the hydrophilic surface. The ABD melting temperatures ranged from 358 K in the worst case to 368 K in the best case, a range of only 10 K. Figure 11 shows a comparison of a more stable case, PRO375, with a less stable case, GLN310. Unlike the hydrophobic surface, the transitions occur at approximately the same temperatures.

IV. DISCUSSION

A. Analysis of hypothesis

The hypothesis tested in this work was that *tethering the antibody at a residue far from the antigen binding domain will yield more stable active sites than when tethers are placed closer*. At first glance, the data seem to support this conclusion. Specifically, the residue on the molecule that produced the most stable ABD when used for tethering on the hydrophilic surface was PRO375. This residue resides in the CH3 region which is the farthest from the ABDs. However, the residue that produced the most stable ABD for the hydrophobic surface was PRO226, which is located in the hinge region. In general, the most stable molecules resulted from tethers placed in the hinge region of the antibody which is very close to the ABDs. The next, most-stable configurations were for tethers placed in the CH3 region where PRO375 is found. The least stable molecule resulted from tethers placed in the CH2 region. Thus, the tether distance from the ABD does not seem to correlate with ABD stability—an observation further supported by the fact that each region of the antibody had residues that, when used to tether the molecule to the surface, both stabilized and destabilized the ABDs.

B. Implications for antibody arrays

The results indicate that tethering to a hydrophobic surface generally destabilizes the antibody. Even the most stable case melted around 10 K lower than the bulk case. As the protein function is directly related to the protein structure, these results suggest that the antibody array performance is likely compromised if the substrate used is hydrophobic.

In contrast, when the antibody is tethered to the hydrophilic surface, it is roughly as stable as the bulk case,

and in some instances, the ABDs were actually stabilized by the presence of the surface. As has been explained in other works, stabilization on the surface occurs because the protein can make the same favorable contacts in the folded state as in the bulk.^{34,37,39,50,60} Specifically, surfaces always stabilize proteins entropically because the surface reduces the entropic cost of folding. [See especially Refs. 60 and 37.] Whether the protein is ultimately stabilized or destabilized, compared to the bulk, depends on the enthalpy of folding. If the surface does not hinder the protein from making the same contacts as in the bulk, the protein will be stabilized. However, if the surface prevents some contacts from being folded, the protein may be destabilized. As depicted in Fig. 10, the Fabs in the hydrophilic case try to keep away from the surface, so it is expected that the change in the enthalpy upon folding will be the same for both the bulk and surface cases. The stabilization is not large for the same reason. Specifically, because the Fabs extend away from the surface, the entropic benefits for surface folding are diminished. The overall effect is that the hydrophilic surface behaves like the bulk, and using hydrophilic surfaces as the substrate for antibody arrays should therefore yield a superior device.

The results also suggest that certain avenues to optimize the array performance will have different effects depending on the type of the surface employed. The hydrophobic cases had a much larger range of melting temperatures than the hydrophilic cases, which can be seen by comparing Figs. 8 and 11. In the hydrophobic case, moving the tethering location caused a noticeable shift in the heat capacity curve, whereas moving the tethering location in the hydrophilic case affected the shape of the curve but did not noticeably shift the peaks. This difference indicates that the tether location is a much stronger function of the location when the antibody is tethered to a hydrophobic surface than to a hydrophilic surface. As such, the destabilizing effect of the hydrophobic surface can be somewhat tempered by tethering the antibody at a more stable location. In short, more care should be taken to correctly place tethers if a hydrophobic surface is required. If a hydrophilic surface is used, less attention needs to be paid for proper tether placement.

ACKNOWLEDGMENTS

The authors are grateful for the funding from the National Science Foundation (NSF) (Grant No. CBET-1054867) and for computer resources from the Fulton Supercomputing Laboratory at Brigham Young University.

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