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Faster Surface Ligation Reactions Improve Immobilized Enzyme Structure and Activity

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Abstract

During integration into materials, the inactivation of enzymes as a result of their interaction with nanometer size denaturing “hotspots” on surfaces represents a critical challenge. This challenge, which has received far less attention than improving the long-term stability of enzymes, may be overcome by limiting the exploration of surfaces by enzymes. One way this may be accomplished is through increasing the rate constant of the surface ligation reaction and thus probability of immobilization with reactive surface sites (i.e., ligation efficiency). Here, the connection between ligation reaction efficiency and the retention of enzyme structure and activity was investigated by leveraging the extremely fast reaction of strained trans-cyclopropenecyclooctenes (sTCOs) and tetrazines (Tet). Remarkably, upon immobilization via Tet-sTCO chemistry, carbonic anhydrase (CA) retained 77% of its solution-phase activity, while immobilization via less efficient reaction chemistries, such as thiol-maleimide and azide-dibenzocyclooctyne, led to activity retention of only 46% and 27%, respectively. Dynamic single-molecule (SM) fluorescence tracking methods further revealed that longer surface search distances prior to immobilization ($>0.5\ \mu\text{m}$) dramatically increased the probability of CA unfolding. Notably, CA distance to immobilization was significantly reduced through the use of tetrazine-sTCO chemistry, which correlated with the increased retention of structure and activity of immobilized CA compared to the use of slower ligation chemistries. These findings provide unprecedented insight into the role of ligation reaction efficiency in mediating the exploration denaturing hotspots on surfaces by enzymes, which, in turn, may have major ramifications in the creation of functional biohybrid materials.

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Supporting Information. Circular dichroism and urea denaturation curves of CA constructs. Contact angle and dry thickness of modified surfaces. Results from SM-FRET experiments, including donor–acceptor intensity histograms, steady state folded fraction, and fraction of un-immobilized CA molecules. Primers, plasmids, and strains used for the creation of CA constructs. Representative movie of raw data for SM-FRET experiments.

Introduction

The creation of biohybrid materials that combine the exquisite specificity and efficiency of enzymes and robust features of materials has opened the door to the use of enzymes in many fields. Specifically, through the merger of enzyme and materials, a world in which enzymes are used in applications as diverse as catalysis^{1,2}, medicine^{3,4}, and bioremediation^{5,6} has become a reality. However, while it is often desirable to exploit the efficiency of enzymes in applications that require synthetic materials and systems, the inactivation of enzymes upon integration into materials remains a major challenge^{7,8}. Additionally, the promise of such biohybrid materials is frequently limited by the fact that enzymes generally function in only a narrow range of conditions. As such, the development of strategies to improve the functional utility of enzymes upon immobilization has been the focus of considerable effort^{9–12}.

Recent findings from dynamic single-molecule experiments have significantly altered our view of how the functional utility of enzymes upon immobilization may be improved^{13–16}. Based on these findings, it has become apparent that both long-term stability and the immediate retention of enzyme activity and structure during immobilization to surfaces are critical. Importantly, because long-term stability and immediate retention of activity and structure involve different mechanisms, different strategies may be required to address each of these aspects. For example, while the long-term stability of immobilized enzymes may be enhanced by tethering enzymes to materials that stabilize the native state of the enzyme and/or promote refolding of denatured enzyme molecules, the immediate retention of activity and structure may be enhanced by controlling the exploration of the surface by the enzyme prior to reaction with the material. Although there has been extensive work on improving the long-term stability of immobilized enzymes^{14,17–19}, preventing the immediate denaturation of enzymes has received far less attention.

In prior studies, we have shown that the unfolding of proteins in contact with materials is often caused by interactions with denaturing sites and is mediated by intermittent diffusion of protein molecules on the material surface¹³. As protein molecules diffuse along the material-solution interface, the molecules may encounter anomalous sites that promote interaction with the unfolded state of the protein. These sites, which can arise from inherent chemical and/or topological irregularities in the surface^{13,20–22}, serve as denaturing “hotspots”. As such, this process involves exploration of local variations in chemical and physical properties of the surface by the protein. This understanding led us to hypothesize that reducing surface diffusion during immobilization may directly lead to significant enhancements in the immediate retention of enzyme structure and thus activity. We therefore sought to investigate this hypothesis by systematically varying the likelihood of ligation of an enzyme molecule with reactive surface sites and thus immobilization efficiency. This approach was enabled by recent developments in bioconjugation reactions with extremely fast ligation reaction rate constants^{23–25}. For example, with the demonstration of novel ligation reactions, including the inverse electron demand Diels–Alder reaction between strained cyclopropene fused trans-cyclooctenes (sTCOs) and tetrazine (Tet), the rate constants of ligation reactions may be systematically varied by several orders of magnitude. Reactions like that between sTCO and Tet are so fast that they have been shown to

enable sub-stoichiometric labeling of proteins under physiological conditions²⁶. The use of such reactions is further enabled by advancements in genetic code expansion that allow bioorthogonal moieties such as TCO and Tet to be installed at precise locations within a protein^{12,27}.

Herein, we leveraged the development of ultrafast bioorthogonal reactions to investigate the connection between ligation efficiency and retention of enzyme structure and activity. This connection was specifically investigated using carbonic anhydrase (CA) as a model enzyme, which was immobilized using three different chemistries, including azide-dibenzocyclooctyne (DBCO), thiol-maleimide, and tetrazine-sTCO coupling. Because the rate constants for these reactions vary over several orders of magnitude, the use of these reactions allowed us to systematically vary the surface ligation efficiency (i.e., the probability of an enzyme molecule reacting with a potential tethering site on the surface). Notably, the reaction constants for azide-DBCO, cysteine-maleimide, and tetrazine-sTCO coupling in aqueous conditions at similar temperature and pH have been reported to be $\sim 10^{-1}$, 10^2 , and $10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively^{28–31}. Systematic comparison of these chemistries was enabled by genetic code expansion such that the ligation site on the CA constructs was constant. Using SM fluorescent tracking methods, the activity and fraction of folded CA for each chemistry was correlated with the search distance of the enzyme prior to immobilization. Our results provide unprecedented insight into the impact of ligation efficiency on the retention of immobilized enzyme activity and structure through mediating enzyme diffusion and therefore controlling the interaction with potential denaturing sites. While demonstrating this impact, these findings have important implications on the development of rational strategies to improve how enzymes are immobilized on materials for the creation of functional biohybrid materials.

Results and Discussion

Impact of Ligation Efficiency on Ensemble Activity and Structure

To test the connection between ligation efficiency (i.e., probability of immobilization with reactive sites) and retention of enzyme structure and activity, three CA constructs were initially created that contained either the amino acid azido-l-phenylalanine (AzF), cysteine, or 3-(6-alkyl-s-tetrazin-3-yl) phenylalanine (Tet3.0) at position 126. As shown in Figure 1A, these constructs, referred to hereafter as CA_{AzF}, CA_{Cys}, and CA_{Tet}, were designed to permit site-specific ligation to either DBCO, maleimide, or sTCO-functionalized silica microparticles (1 μm mean diameter), via slow, intermediate or fast ligation reaction rate constant, respectively. Residue position 126 was chosen because it is remote from the active site, which would reduce the probability that immobilization would result in obstruction of the active site¹². Importantly, to generate CA_{AzF}, CA_{Cys}, and CA_{Tet}, we used the thermally stable variant C205S in which the only native cysteine in CA was replaced with a serine. Characterization of the constructs via circular dichroism showed that the amino acid substitutions had negligible effect on the secondary structure of the enzyme compared to wild-type CA (Figure S1). Additionally, urea denaturation curves using tryptophan fluorescence showed the stability of the constructs was similar based on the concentration of urea required to unfold 50% of CA (Figure S2).

Enzyme immobilization was enabled via modifying silica microparticles with epoxide groups, which were subsequently reacted with DBCO-amine, maleimide-amine, or sTCO-amine. Upon immobilization to the modified particles, the specific activity of CA_{AzF}, CA_{Cys}, and CA_{Tet} was determined, relative to the activity of the soluble form of each construct. Interestingly, relative specific activity varied significantly based on the ligation chemistry used and increased significantly with increasing ligation efficiency (Figure 1B). Specifically, when immobilized on DBCO functionalized microparticles via the least efficient AzF-DBCO chemistry, the specific activity of CA_{AzF} relative to CA_{AzF} in solution was only 0.27 ± 0.04 . On maleimide surfaces, where ligation occurred via the more efficient thiol-maleimide chemistry, the activity retention of CA_{Cys} was higher (0.46 ± 0.06). Finally, on the sTCO surfaces, after immobilization via the most efficient tetrazine-sTCO chemistry, the specific activity of CA_{Tet} was the highest at 0.77 ± 0.03 . Given the ligation site in each construct of CA was the same, differences in the impact of active site accessibility on activity retention of the constructs was presumably negligible. Additionally, the extent of enzyme loading on the particles was similar for all three constructs (6.2–7.2 mg enzyme per g particles, as shown in Figure S3), thereby negating differential effects of enzyme crowding. To probe the possible effects of underlying surface chemistry, silica wafers were modified with DBCO, maleimide, and sTCO in the same way as the particles and characterized using ellipsometry contact angle goniometry. The modified wafers exhibited dry thicknesses of 1.1 ± 0.1 , 1.06 ± 0.09 and 1.1 ± 0.1 nm and water contact angles of $57^\circ \pm 2$, $57^\circ \pm 2$ and $59.4^\circ \pm 0.7$ for the functionalized DBCO, Maleimide and sTCO surfaces, respectively (Figure S4). Given that all three surfaces had similar contact angles, it is unlikely that differences in relative specific activity between the constructs could be explained by variations in hydrophobicity between the surfaces. Additionally, by using the same chemistry (i.e., epoxide-amine) to introduce DBCO, maleimide, and sTCO groups, variation in the density of ligation sites between surfaces was minimized. Ultimately, these results were consistent with the hypothesis that increased ligation reaction rate constants better preserved the structure and activity of CA following surface ligation.

To determine if the differences in relative specific activity of immobilized CA_{AzF}, CA_{Cys}, and CA_{Tet} were related to the retention of enzyme structure, the average extent of unfolding of the constructs was measured by tryptophan fluorescence. Specifically, by comparing tryptophan fluorescence for each of the immobilized constructs to that of its soluble counterpart in the presence of urea, the apparent fraction of folded CA_{AzF}, CA_{Cys}, and CA_{Tet} immobilized on the microparticles was determined. Interestingly, Figure 1C shows a similar trend for the apparent folded fraction as a function of ligation efficiency as was observed for specific activity. Specifically, the apparent folded fraction was the highest for CA immobilized via sTCO chemistry (0.87 ± 0.02), which is the fastest reaction chemistry, followed by maleimide (0.82 ± 0.02), and DBCO (0.76 ± 0.01), which is the slowest reaction chemistry. This observation supports our hypothesis that more efficient ligation reduces the probability of an enzyme encountering denaturing hotspots on surfaces prior to immobilization. In particular, as the efficiency of ligation increases, the enzyme is presumably less likely to encounter a denaturing hotspot prior to immobilization due to a decrease in the distance traversed by the enzyme before surface ligation. In the case of less efficient ligation, the search distance prior to successful immobilization will

effectively increase given that the enzyme will require numerous interactions with potential ligation sites before successfully reacting. Because the denaturing hotspots are presumably nanometer or sub-nanometer in size, and may involve chemical or physical characteristics, it is difficult to characterize the nature of these sites, or to distinguish benign from denaturing sites using independent structural characterization methods. In the absence of such methods, we have previously confirmed the presence of anomalous surface sites via super-resolution mapping using molecular probes, including solvatochromic dyes^{18,32,33}. These sites may arise from variations in surface roughness as well as local variations in the ratio of hydrophilic siloxane and hydrophobic silanol groups, which are present in fused silica²⁰.

Preparation of Constructs for SM FRET

In light of the results of activity and structural measurements, we sought to characterize the effects of ligation efficiency on the search distance of CA prior to immobilization and unfolding. The impact of ligation efficiency on the search distance for CA prior to immobilization and unfolding was quantified via SM tracking and intramolecular Förster resonance energy transfer (FRET)^{13,34}. Using SM tracking, the molecular trajectories of a large number of individual CA molecules as they diffused on the surface was monitored. While monitoring their diffusion, we were also able to determine when and where each enzyme molecule became immobilized. Additionally, by concurrently measuring intramolecular FRET (which encoded information about molecular conformation), the instantaneous folding state of each molecule throughout its trajectories, including before and after surface ligation, was determined.

SM-FRET tracking was enabled by engineering labeling sites for the attachment of donor (AlexaFluor 555) and acceptor (CF640) fluorophores in CA_{AzF}, CA_{Cys}, and CA_{Tet} at residue positions 20 and 233 (Figure S5), respectively. At these positions, AzF, Cys, or Tet3.0 was introduced such that each construct contained a combination of two of the three amino acids for labeling, where the third amino acid was employed for the site-specific ligation chemistry at position 126 (e.g., for CA_{AzF}, Tet3.0 was introduced at residue 20 and Cys at residue 233, thus maintaining the ability for site specific ligation through the azido-l-phenylalanine residue at position 126). Critical to this approach was the simultaneous incorporation of AzF and Tet3.0 (i.e., two distinct unnatural amino acids) via suppression of two blank codons (TAG and TAA) in *Escherichia coli* using a set of mutually orthogonal aminoacyl-transfer RNA synthetase/tRNA pairs³⁵. To confirm that all three amino acids were present and reactive, the constructs were reacted with a tetrazine-reactive sTCO-bearing polyethylene glycol polymer (M_w 5000), an azide-reactive DBCO-bearing TAMRA, and thiol-reactive methanethiosulfonate-bearing BODIPY. As shown in Figure S6, the triple mutant constructs designed for FRET showed signatures of modification with all three reagents via in-gel SDS-PAGE imaging. Furthermore, the sensitivity of the FRET signature to unfolding after labeling with AlexaFluor 555 and CF640 was shown by measuring FRET of the constructs in solution in the presence of urea. As shown in Figure S7, the FRET signature of all three constructs decreased significantly with increasing concentration of urea, which is consistent with unfolding.

Analysis of SM Surface Trajectories

For SM-FRET experiments, the surfaces were exposed to labeled CA in a flow cell and individual enzyme molecules were observed via wide-field total internal reflection fluorescence microscopy. Localization of enzyme molecule upon introduction to the flow cell was enabled via rapid alternating excitation of the donor and acceptor fluorophores (see Movie S1 as a sample of raw data from SM-FRET experiments, which shows CA in contact with the sTCO-modified surface). From the time-dependent FRET emission of the donor and acceptor, the instantaneous conformation (i.e., folded versus unfolded) of each CA molecule was determined at every point in its trajectory. Criteria for distinguishing the folding state of molecules was established from analysis of donor and acceptor emission for all observations for each surface. Specifically, distributions like that shown in Figure S8 were generated from the accumulation of 5,100–8,900 trajectories and were further used to assign the folding state of each molecular observation. In addition to identifying folding state, individual trajectories were segmented based on the point in time in which immobilization to the surface occurred. Immobilization was considered to have occurred if the position of a molecule was within tracking error of the position of the final frame in its trajectory for at least three consecutive frames and remained within localization error throughout the remaining of the trajectory.

Representative temporal and spatial trajectories of labeled CA in contact with the surface of silica wafers modified with DBCO, maleimide, and sTCO are shown in Figure 2. As illustrated in the representative trajectories, the extent of conformational fluctuations and the length of the trajectories prior to surface ligation varied significantly between molecules. For example, some molecules remained folded over the entire lifetime of their trajectory and engaged in a brief local search before successful immobilization (Trajectory 1, panels A and D). In other cases, molecules underwent a more extensive search and appeared to unfold prior to surface immobilization (Trajectory 2, panels B and E). As a final example, there were also cases where a molecule unfolded while diffusing on the surface and eventually desorbed from the surface without reacting with the surface (Trajectory 3, panels C and F). As observed previously, the surface trajectories of CA molecules prior to ligation consisted of intermittent periods of confinement and short and long flights^{13,36–38}. Such “hopping” diffusion can be described as a generalized continuous time random walk process, where the periods of confinement represent time intervals when a molecule is adsorbed strongly to the surface and the flights represent periods when the molecule effectively “hops” along the surface, thereby sampling the local environment until eventually re-adsorbing³⁹. This motion is illustrated in the two-dimensional representation of the molecular trajectories of individual CA molecules in panels G, H, and I. As such, the data in the trajectories provided information about whether each molecule was searching or immobilized, and also if a molecule was folded or unfolded at any given time while in contact with the surface.

Connecting Single-Molecule Trajectories and Unfolding

Analysis of SM-FRET trajectories enabled the correlation between ligation efficiency, search distance, and folding state prior to immobilization to be directly observed. Importantly, we found that more efficient ligation chemistries correlated with shorter trajectories prior to surface ligation and a lower probability of unfolding prior to

immobilization. These phenomena are visualized qualitatively in Figure 3, using randomly selected surface trajectories (between 25–30 frames long) of molecules that were initially folded and became immobilized during their trajectory. Notably, based on the apparent overall size of the trajectories in Figure 3, it is apparent that the region of the surface searched by CA decreased systematically from DBCO to maleimide surfaces, and were the most compact on sTCO surfaces, which had the most efficient ligation. On DBCO surfaces, where the ligation efficiency was lowest, many trajectories exhibited local searching followed by one or more long flights prior to immobilization. On maleimide surfaces, the fraction of trajectories exhibiting long flights was substantially reduced, and on sTCO surfaces, where the ligation efficiency was greatest, the majority of trajectories were immobilized within the initial searching interval, and never exhibited a long flight.

Additionally, by segmenting the surface trajectories based on folding state (red vs. black line segments), a clear trend between the length of surface trajectories prior to immobilization and unfolding of CA was observed. Specifically, as the overall size of surface trajectories decreased (due to more rapid and efficient ligation), the fraction of CA molecules that unfolded was reduced considerably. This is particularly evident by comparing the fraction of trajectories that underwent unfolding on the DBCO and sTCO surfaces (red line segments in Figures 3A and 3C, respectively). Further analysis of the trajectories revealed that unfolding frequently occurred after long step displacements, suggesting re-adsorption on denaturing sites after a flight¹³. Given the correlation between increased ligation efficiency and shorter search distances and less unfolding prior to ligation, these findings support the hypothetical connection between reduced surface exploration and increased activity retention during surface immobilization.

To quantify the observations described above, the distribution of distance prior to immobilization for CA was calculated. In particular, for each surface, the Euclidian distance prior to immobilization was analyzed for 1200–2000 surface trajectories. Figure 4A shows that on all functionalized surfaces, the distribution of Euclidian distance prior to immobilization exhibits a peak at $\sim 0.2 \mu\text{m}$, and that the majority of CA molecules were immobilized within $\sim 0.4 \mu\text{m}$ of the location where they initially adsorbed to the surface. Notably, the peak centered at $\sim 0.2 \mu\text{m}$ represents the population of CA molecules that were immobilized within their initial exploration time interval following adsorption. However, the immobilization distributions on the DBCO and maleimide surfaces exhibited a distinct shoulder extending to longer distances (centered at $\sim 0.9 \mu\text{m}$), demonstrating the presence of a second population (see inset of Figure 4A). This second population represents CA molecules that failed to react with a potential immobilization site during their initial searching period and therefore underwent one or more flights before becoming immobilized during a subsequent searching period. These distributions are similar to those observed for the surface-mediated hybridization of DNA on surfaces with low grafting densities of complementary DNA⁴⁰.

The fraction of immobilized molecules in the first and second populations were quantified by fitting the distributions to a log-normal mixture model, chosen to capture the heavy tail that characterized the populations. As expected, the fraction of immobilization events in the first population decreased systematically with decreasing ligation efficiency, being

highest on sTCO (0.938 ± 0.007) followed by on maleimide (0.87 ± 0.05) and then on DBCO (0.83 ± 0.01) functionalized surfaces. Conversely, the second population was most prominent on DBCO and maleimide surfaces with fractions of 0.17 ± 0.02 and 0.13 ± 0.06 , respectively. The second populations on the sTCO surface accounted for only 0.06 ± 0.01 of the total number of CA molecules. These results quantitatively demonstrate that by using a more efficient ligation chemistry, the probability of enzyme molecules undergoing subsequent flights after the initial surface adsorption was reduced, which may in turn lower the probability of encountering denaturing sites upon re-adsorption. Although CA_{cys} may undergo the reverse retro-Michael reaction, the kinetics for the retro-Michael reaction are considerably slower than the forward Michael addition at the conditions used^{41,42}. Given the timescale of the retro-Michael reaction relative to the Michael addition, it is unlikely the reverse reaction affected the interpretation of our SM-FRET results.

The statistical differences between the distance to immobilization for different surface chemistries was further visualized using semi-logarithmic plots of complementary cumulative distributions (CCCDs) of the Euclidean distance to immobilization (Figure 4B). The CCCD represents the fraction of enzymes remaining un-immobilized after traveling a given distance from the initial adsorption site. Consistent with the qualitative trend from Figure 3 (and the shoulders of the raw distributions in Figure 4A), the CCCD associated with sTCO surfaces decayed most rapidly with distance followed by maleimide and DBCO surfaces, in that order. Specifically, on sTCO surfaces with the fastest ligation reaction rate constant, 90% of the molecules were immobilized within $0.45 \mu\text{m}$ of the location of the first frame of the trajectory, whereas on maleimide and DBCO surfaces with intermediate and slow ligation reaction rate constants, respectively, this distance was $0.70 \mu\text{m}$ and $0.83 \mu\text{m}$, respectively. Notably, some molecular trajectories were “unsuccessful” and did not end in immobilization. In particular, some enzymes underwent extremely long flights, desorbed completely, or photobleached prior to surface ligation. Interestingly, the percent of enzymes that were not immobilized increased significantly with decreasing ligation efficiency. As shown in Figure S9, on sTCO surfaces, the fraction of molecules remained un-immobilized over their entire trajectories was only 0.058 ± 0.007 , while on maleimide and DBCO surfaces, where immobilization occurred via slower ligation reaction rate constants, the fraction of molecules that remained un-immobilized was 0.10 ± 0.01 and 0.147 ± 0.008 , respectively.

Despite the impact of ligation efficiency on search distance, the fraction of folded CA at the initial point of immobilization was independent of ligation efficiency. Specifically, analysis of the fraction of folded CA at the initial point of immobilization indicated the fraction of folded CA as a function of search distance was the same for all three chemistries (Figure 4C). As evident from this analysis, CA molecules immobilized within $\sim 0.4 \mu\text{m}$ of their adsorption locations regardless of the ligation chemistry were predominantly folded whereas the fraction of folded molecules decrease sharply as the search distance increased. Interestingly, this critical distance corresponded to the distance within which the majority of molecules became immobilized during their initial searching period (i.e., without undergoing a flight) (Figure 4A). As such, this indicates that molecules that underwent one or more flights had a higher likelihood of unfolding prior to immobilization, which is

consistent with previous observations¹³. Importantly, these findings confirm that the primary factor in the retention of CA structure was indeed search distance.

In addition to characterizing the dynamics of CA molecules prior to immobilization, the steady-state folded fraction of CA molecules after immobilization was measured using SM-FRET. As shown in Figure S10, the folded fraction of CA enzymes immobilized on sTCO (0.81 ± 0.04) surfaces, via fast reaction ligation rate constant, was significantly higher compared to on maleimide (0.56 ± 0.03), and DBCO (0.37 ± 0.04) surfaces, where the ligation reaction rate constants were slower. Although more pronounced, this trend in folded fraction with ligation efficiency is consistent with the trend measured in ensemble-averaged experiments using tryptophan fluorescence (Figure 1C). The trend from SM-FRET may be more pronounced due to the difference in sensitivity of SM-FRET compared to tryptophan fluorescence to changes in local CA conformation. Notably, while small changes in local conformation may be detected by SM-FRET, such changes may not significantly alter the solvent environment around tryptophan residues.

Interestingly, the observations shown here may also explain prior results where non-specific tethering led to an increase in the retention of folded enzyme compared to site-specific tethering⁴³. Specifically, our findings are consistent with the hypothesis that non-specific tethering is more efficient than site-specific tethering since the number of possible tethering sites is greater. In this case, the number of tethering sites is greater for non-specific immobilization because there is not a requirement that the enzyme be in a specific orientation relative to the surface to react. As such, the apparent increase in retention of enzyme structure is likely due to a decrease in the probability of the enzyme encountering a denaturing hotspot prior to successful immobilization. Although not investigated here, the immobilization efficiency for a given ligation chemistry may also be enhanced by increasing the density of reactive sites on the surface of the material. In particular, an increase in surface ligation sites could decrease the overall search distance until immobilization, leading to higher structure and activity retention. Interestingly, this is supported by previous results from Betancor and co-workers⁴⁴, where higher activity retention of immobilized glucose oxidase was observed on supports functionalized with dimeric glutaraldehyde than with monomeric glutaraldehyde, likely due to the higher number of glutaraldehyde reactive groups used for ligation. This approach could be complementary to the use of more efficient ligation chemistries. Finally, our findings may also provide an alternative or complementary explanation for the apparent increase in activity of tethered enzymes on charged immobilization supports^{45,46}. Specifically, the formation of ionic interactions prior to surface ligation may restrict enzyme diffusion, thereby reducing the extent of searching.

Conclusions

Our findings demonstrate the retention of CA activity and structure is strongly dependent on the efficiency of ligation chemistry. The connection between CA activity and structure and the efficiency of ligation chemistry was shown by combining ensemble activity and structure measurements and SM-FRET analysis. Using SM-FRET analysis, we specifically showed that increased ligation efficiency resulted in less exploration of the surface by CA as well as ultimately less unfolding prior to immobilization. Importantly, by limiting

exploration of the surface, an enzyme is presumably less likely to encounter anomalously strong denaturing sites that may be present on the surface, e.g., arising from surface defects. Given these findings, our results suggest that the functional utility of immobilized enzymes may be greatly improved by using ultrafast ligation reactions to conjugate enzymes to materials. Although our observations highlight how the immediate retention of immobilized enzyme activity and structure may be improved, other methods may be necessary to improve long-term stability. In light of this, it is interesting to consider how ultrafast ligation methods may be combined with methods to improve long-term stability such as the use of materials as immobilization supports that stabilize the native state of the enzyme and/or promote refolding of denatured enzyme molecules. A promising approach may also entail combining ultrafast ligation methods with multipoint covalent immobilization. In this case, rather than incorporation of a tetrazine-containing amino acid in one location within the enzyme, tetrazine-containing amino acids may be incorporated in multiple locations. As such, while enabling the enzyme to react with the surface rapidly, multiple linkages between the enzyme and material may be formed, which can lead to an increase in enzyme rigidity. Of further interest is to study the generality of the relationship between immobilization efficiency and enzyme stability and activity in the context of other classes of enzyme. Ultimately, the understanding from this work may thus form the basis of a plethora of strategies for the rational design of biohybrid materials. Moreover, such understanding can be used to improve the homogeneity of surfaces containing immobilized proteins, which can facilitate characterization of surface attached proteins.

Materials and Methods

Surface Preparation and Characterization

For SM imaging and ellipsometry measurements, two-inch fused silica wafers (Mark Optics) and silicon wafers with native oxide coating (Wafer Pro) were cleaned in piranha solution (30% v/v hydrogen peroxide, 70% sulfuric acid) for 1 h, rinsed with water, dried under nitrogen, and subsequently cleaned for 1 h with UV-ozone prior to use. For ensemble activity and tryptophan fluorescence measurements, silica microparticles (NanoCym, 1 μm diameter) were cleaned via UV-ozone for 1 h prior to functionalization. The particles were functionalized via vacuum assisted chemical vapor deposition with a solution of n-butylamine:3-glycidoxypropyltrimethoxysilane:toluene at a 1:2:20 ratio by volume⁴⁷. During vapor deposition, the solution, wafers, and microparticles were all placed in a desiccator for 24 h. Surfaces were then rinsed with toluene, iso-propanol, and water, in that order. Glycidoxypropyltrimethoxysilane-modified surfaces were then placed in an ethanol solution containing 1 mM DBCO-amine (Sigma-Aldrich), 1-(2-Aminoethyl)maleimide (Sigma-Aldrich), or sTCO-amine (synthesized as described previously⁴⁸) and allowed to react for 24 h. Surfaces were finally rinsed with ethanol and water and dried with nitrogen gas. To confirm functionalization, wafers were characterized via measuring static water contact angles using a custom-built goniometer and via measuring dry thickness using a variable-angle spectroscopic ellipsometer (VASE-VB-250). Finally, supported lipid bilayers were composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine lipids and were formed as described previously⁴⁹.

Cloning, Expression, and Purification of CA constructs

CA constructs (Table S1) were cloned from a C205S thermal stable variant using methods that were previously described elsewhere¹². Following cloning, CA constructs were produced using the DEAL approach as we've previously described³⁵. Briefly, CA constructs were paired with AzF- and Tet3.0-encoding machinery plasmids in *Escherichia coli* BL21(DE3) cells to generate the strains listed in Table S1. These strains were grown overnight in non-inducing media⁵⁰, which was used to inoculate 50 mL of autoinduction media supplemented with 200 μ M ZnSO₄, 0.05% (w/v) arabinose, 0.02% (w/v) lactose, appropriate antibiotics at a 1% (v/v) inoculum, and 500 μ M Tet3.0 or 1 mM AzF (when necessary). After a growth period of 24 h in autoinduction media, cultures were pelleted at 5,000 rcf and lysed by microfluidization. After centrifugation at 21,000 rcf for 30 min, CA in the soluble fraction was purified using Ni-TALON resin. Upon binding of CA, the resin was washed with 50 mM Na₂HPO₄, 500 mM NaCl, 5 mM imidazole, pH 7.0 and CA was eluted with 50 mM Na₂HPO₄, 500 mM NaCl, 250 mM imidazole, pH 7.0. Prior to further use, purified CA was desalted into buffer containing 200 mM HEPES, 150 mM NaCl, 1 μ M ZnSO₄, pH 7.5. Protein concentration was determined by measuring absorbance at 280 nm using the molar extinction coefficient of 61,724 M⁻¹ cm⁻¹ for Tet3.0-containing constructs and 50,070 M⁻¹ cm⁻¹ for constructs without Tet3.0. The purified proteins were aliquoted, flash frozen in nitrogen, and stored at -80°C until needed.

Immobilization of CA constructs

For ensemble activity and stability measurements, CA constructs were immobilized by adding 5 mg of modified microparticles to a 50 μ L solution of 50 μ M CA (100 mM HEPES, 150 mM NaCl, pH 7.5). The immobilization reaction was incubated at room temperature with gentle mixing for 24 h to ensure complete conjugation. Notably, the constructs were stable under these conditions over this period and did not lose any activity. After immobilization, the particles were centrifuged at 1000xg for 2 min and the supernatant was collected after which the particles were resuspended again in fresh HEPES buffer. This procedure was repeated a total of three times. The concentration of residual enzyme in the supernatant that was collected from the wash steps was quantified via measuring absorbance at 280 nm. By means of a mass balance, this concentration was used to determine the amount of enzyme immobilized to the particles. For SM-FRET experiments, FRET-labeled CA constructs were immobilized to functionalized silicon wafers *in situ* via flowing the enzyme over the wafer surface in a custom-built flow cell (internal volume 0.24 mL). To prevent variations in the ligation efficiency due to differences in the concentrations and transport of CA across experiments, all FRET-CA constructs were introduced at the same concentration (5×10^{-11} M) using a syringe pump (Chemyx Inc.) at a constant flow rate of 50 μ L/min for 8 min while collecting movies. To measure the steady-state folded fraction, additional movies were taken after any enzyme that was not covalently immobilized to the wafer surface was purged from the flow cell with buffer for 8 min.

CA Activity Measurements

The activity of CA constructs was assayed using a proprietary commercial ester substrate (BioVision) that upon conversion by CA releases *p*-nitrophenol, which was monitored via

UV/vis spectrometry. Briefly, 2 μL of the substrate was mixed with 10 μL of CA (4.8 μM) and 88 μL of 100 mM HEPES buffer containing 150 mM NaCl and 50 μM ZnSO_4 (pH 7.5). The activity was measured by recording the absorption of *p*-nitrophenol at 405 nm using an Infinite M Plex plate reader (Tecan). Background hydrolysis was subtracted by measuring the release of *p*-nitrophenol in identical conditions in the absence of enzyme. Relative activity was determined by normalizing the activity of the immobilized form of each CA variant to the activity of the same variant in solution.

Tryptophan Fluorescence Measurements

Tryptophan fluorescence was measured at room temperature by exciting 5 μM CA (soluble or immobilized) at 295 nm and collecting the emission intensity between 325–370 nm using an Infinite M Plex plate reader (Tecan). The ratio of the emission intensities at 332 nm to 352 nm^{51,52} for immobilized CA ($F_{332/352, \text{immob}}$) was converted to an apparent fraction of folded CA using a urea denaturation curve for each soluble construct. Urea denaturation curves were similarly measured by tryptophan fluorescence upon incubation of soluble CA with 0, 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7.4, and 7.7 M urea for 24 h at room temperature. Using the denaturation curves, the apparent folded fraction for immobilized CA was calculated using the following equation:

$$\text{Folded Fraction}_i = \frac{F_{332/352, \text{immob}, i} - F_{332/352, \text{free}, 7.7\text{M}, i}}{F_{332/352, \text{free}, 0\text{M}, i} - F_{332/352, \text{free}, 7.7\text{M}, i}}$$

where, $F_{332/352, \text{immob}, i}$, $F_{332/352, \text{free}, 0\text{M}, i}$ and $F_{332/352, \text{free}, 7.7\text{M}, i}$ are the fluorescence intensity ratios of immobilized CA_{*i*}, soluble CA_{*i*} in 0 M urea, and soluble CA_{*i*} in 7.7 M urea, respectively. For all measurements, background fluorescence was measured and subtracted for identical conditions without immobilized or free CA.

SM-FRET Experiments

SM-FRET analysis of immobilized CA structure and dynamics was enabled by labeling CA constructs with donor and acceptor fluorophores. For site-specifically labeling, 50 μL of each CA variant (50 μM) was incubated with AlexaFluor 555 (Life Technologies) and CF 640 (Biotium) dyes at 5:1 ratio of each fluorophore-to-enzyme in labeling buffer (100 mM HEPES, 150 mM NaCl, pH 7.5) for 24 h at room temperature in the dark. The specific construct/dye chemistry combinations were prepared as described in Figure S4. Excess dye was removed by serial washing the labeled enzyme three times with labeling buffer using a Zeba spin column with a 7K MWCO (Thermo Fisher). Labeling efficiencies were determined using the molar extinction coefficient for each dye and the theoretical adsorption coefficient of each of the constructs at 280 nm. The labeling efficiency, after correcting for absorbance of the dyes at 280 nm, for AlexaFluor 555 was 56%, 38%, and 58% for CA_{AZF}, CA_{Cys}, and CA_{Tet}, respectively, while the labeling efficiency for CF 640 was 72%, 56%, and 50% for CA_{AZF}, CA_{Cys}, and CA_{Tet}, respectively.

SM-FRET trajectories were subsequently collected at room temperature via alternating laser excitation with a 100 ms acquisition time using a custom-built prism-based TIRF microscope with a 50 mW 532 nm DPSS laser (Cobolt, Samba) and a 50 mW 640 nm

DPSS laser (Crystalaser). Emission intensities from labeled enzymes were separated using a 610 nm dichroic mirror (Chroma). Donor and acceptor emissions were further separated with 585/40 and 685/40 bandpass filters (Semrock) before images were collected by an Andor iXon3 888 EMCCD camera. A custom MATLAB software was used for object tracking, intensity quantification, and instantaneous folding state identification as described previously^{32,49}. Additionally, for identification of immobilization state, the location of CA at each frame was compared to the location during the final frame of the trajectory. Enzyme molecules were deemed immobilized if the position of CA was within tracking error of the position at the final frame for at least three consecutive frames and continued to be within tracking error for the remaining of the trajectory. Finally, probability density distributions of the Euclidian distance to immobilization were fitted to a log-normal mixture model,

$$P(x) = \sum_i \left(\frac{f_i}{\sqrt{2\pi}\sigma_i x} \right) \exp\left(-\frac{(\ln x - \mu_i)^2}{2\sigma_i^2}\right), \text{ with } f_j \text{ representing the fraction of enzymes in population } i \text{ having shape parameter } \sigma_j \text{ and position parameter } \mu_j.$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- (1). Bernal C; Rodríguez K; Martínez R Integrating Enzyme Immobilization and Protein Engineering: An Alternative Path for the Development of Novel and Improved Industrial Biocatalysts. *Biotechnology Advances*. 2018, 36 (5), 1470–1480. [PubMed: 29894813]
- (2). Zhang Y; Yue Q; Zagho MM; Zhang J; Elzatahry AA; Jiang Y; Deng Y Core-Shell Magnetic Mesoporous Silica Microspheres with Large Mesopores for Enzyme Immobilization in Biocatalysis. *ACS Appl. Mater. Interfaces* 2019, 11 (10), 10356–10363. [PubMed: 30789700]
- (3). Aimetti AA; Machen AJ; Anseth KS Poly(Ethylene Glycol) Hydrogels Formed by Thiol-Ene Photopolymerization for Enzyme-Responsive Protein Delivery. *Biomaterials* 2009, 30 (30), 6048–6054. [PubMed: 19674784]
- (4). Rocchitta G; Spanu A; Babudieri S; Latte G; Madeddu G; Galleri G; Nuvoli S; Bagella P; Demartis MI; Fiore V; Manetti R; Serra PA Enzyme Biosensors for Biomedical Applications: Strategies for Safeguarding Analytical Performances in Biological Fluids. *Sensors* 2016, 16 (6), 780.
- (5). Russell AJ; Berberich JA; Drevon GF; Koepsel RR Biomaterials for Mediation of Chemical and Biological Warfare Agents. *Annual Review of Biomedical Engineering* 2003, 5 (1), 1–27.
- (6). Sharma B; Dangi AK; Shukla P Contemporary Enzyme Based Technologies for Bioremediation: A Review. *Journal of Environmental Management* 2018, 210, 10–22 [PubMed: 29329004]
- (7). Hoarau M; Badiéyan S; Marsh ENG Immobilized Enzymes: Understanding Enzyme – Surface Interactions at the Molecular Level. *Org. Biomol. Chem* 2017, 15 (45), 9539–9551. [PubMed: 28932860]

- (8). Jia F; Narasimhan B; Mallapragada S Materials-Based Strategies for Multi-Enzyme Immobilization and Co-Localization: A Review. *Biotechnol. Bioeng* 2014, 111 (2), 209–222. [PubMed: 24142707]
- (9). Silva C; Martins M; Jing S; Fu J; Cavaco-Paulo A Practical Insights on Enzyme Stabilization. *Crit. Rev. Biotechnol* 2018, 38 (3), 335–350. [PubMed: 28764566]
- (10). Rueda N; dos Santos JCS; Ortiz C; Torres R; Barbosa O; Rodrigues RC; Berenguer-Murcia Á; Fernandez-Lafuente R Chemical Modification in the Design of Immobilized Enzyme Biocatalysts: Drawbacks and Opportunities. *Chem. Rec* 2016, 16 (3), 1436–1455. [PubMed: 27166751]
- (11). Garcia-Galan C; Berenguer-Murcia Á; Fernandez-Lafuente R; Rodrigues RC Potential of Different Enzyme Immobilization Strategies to Improve Enzyme Performance. *Advanced Synthesis and Catalysis*. John Wiley & Sons, Ltd 11 1, 2011, 353 (16), 2885–2904.
- (12). Bednar RM; Golbek TW; Kean KM; Brown WJ; Jana S; Baio JE; Karplus PA; Mehl RA Immobilization of Proteins with Controlled Load and Orientation. *ACS Appl. Mater. Interfaces* 2019, 11 (40), 36391–36398. [PubMed: 31525993]
- (13). Weltz JS; Schwartz DK; Kaar JL Surface-Mediated Protein Unfolding as a Search Process for Denaturing Sites. *ACS Nano* 2015, 10 (1), 730–738. [PubMed: 26580418]
- (14). Chaparro Sosa AF; Black KJ; Kienle DF; Kaar JL; Schwartz DK Engineering the Composition of Heterogeneous Lipid Bilayers to Stabilize Tethered Enzymes. *Adv. Mater. Interfaces* 2020, 7 (17), 2000533.
- (15). Kienle DF; Chaparro Sosa AF; Kaar JL; Schwartz DK Polyelectrolyte Multilayers Enhance the Dry Storage and PH Stability of Physically Entrapped Enzymes. *ACS Appl. Mater. Interfaces* 2020, 12 (20), 22640–22649. [PubMed: 32352745]
- (16). Weltz JS; Kienle DF; Schwartz DK; Kaar JL Dramatic Increase in Catalytic Performance of Immobilized Lipases by Their Stabilization on Polymer Brush Supports. *ACS Catal.* 2019, 9 (6), 4992–5001.
- (17). Swartzlander MD; Barnes CA; Blakney AK; Kaar JL; Kyriakides TR; Bryant SJ Linking the Foreign Body Response and Protein Adsorption to PEG-Based Hydrogels Using Proteomics. *Biomaterials* 2015, 41, 26–36. [PubMed: 25522962]
- (18). Faulón Marruecos D; Saleh LS; Kim HH; Bryant SJ; Schwartz DK; Kaar JL Stabilization of Fibronectin by Random Copolymer Brushes Inhibits Macrophage Activation. *ACS Appl. Bio Mater* 2019, 2 (11), 4698–4702.
- (19). Wu J; Zhao C; Lin W; Hu R; Wang Q; Chen H; Li L; Chen S; Zheng J Binding Characteristics between Polyethylene Glycol (PEG) and Proteins in Aqueous Solution. *J. Mater. Chem. B* 2014, 2 (20), 2983–2992. [PubMed: 32261674]
- (20). Bolis V; Cavenago A; Fubini B Surface Heterogeneity on Hydrophilic and Hydrophobic Silicas: Water and Alcohols as Probes for H-Bonding and Dispersion Forces. *Langmuir* 1997, 13 (5), 895–902.
- (21). Pingle H; Peng-Yuan W; McArthur S; Kingshott P Manufacture of Chemically Modified Antibacterial Surfaces. *Antibacterial Surfaces* 2015, 61–88.
- (22). Bull DS; Kienle DF; Chaparro Sosa AF; Nelson N; Roy S; Cha JN; Schwartz DK; Kaar JL; Goodwin AP Surface-Templated Nanobubbles Protect Proteins from Surface-Mediated Denaturation. *J. Phys. Chem. Lett* 2019, 10 (11), 2641–2647. [PubMed: 31067058]
- (23). Wang P; Na Z; Fu J; Tan CYJ; Zhang H; Yao SQ; Sun H Microarray Immobilization of Biomolecules Using a Fast Trans-Cyclooctene (TCO)—Tetrazine Reaction. *Chem. Commun* 2014, 50 (80), 11818–11821.
- (24). Vinogradova EV; Zhang C; Spokoyny AM; Pentelute BL; Buchwald SL Organometallic Palladium Reagents for Cysteine Bioconjugation. *Nature* 2015, 526 (7575), 687–691. [PubMed: 26511579]
- (25). Zheng M; Zheng L; Zhang P; Li J; Zhang Y Development of Bioorthogonal Reactions and Their Applications in Bioconjugation. *Molecules* 2015, 20 (2), 3190–3205. [PubMed: 25690284]
- (26). Blizzard RJ; Backus DR; Brown W; Bazewicz CG; Li Y; Mehl RA Ideal Bioorthogonal Reactions Using a Site-Specifically Encoded Tetrazine Amino Acid. *J. Am. Chem. Soc* 2015, 137 (32), 10044–10047. [PubMed: 26237426]

- (27). Lang K; Davis L; Wallace S; Mahesh M; Cox DJ; Blackman ML; Fox JM; Chin JW Genetic Encoding of Bicyclononynes and Trans-Cyclooctenes for Site-Specific Protein Labeling in Vitro and in Live Mammalian Cells via Rapid Fluorogenic Diels-Alder Reactions. *J. Am. Chem. Soc* 2012, 134 (25), 10317–10320. [PubMed: 22694658]
- (28). Darko A; Wallace S; Dmitrenko O; Machovina MM; Mehl RA; Chin JW; Fox JM Conformationally Strained Trans-Cyclooctene with Improved Stability and Excellent Reactivity in Tetrazine Ligation. *Chem. Sci* 2014, 5 (10), 3770–3776. [PubMed: 26113970]
- (29). Marks IS; Kang JS; Jones BT; Landmark KJ; Cleland AJ; Taton TA Strain-Promoted “Click” Chemistry for Terminal Labeling of DNA. *Bioconjug. Chem* 2011, 22 (7), 1259–1263. [PubMed: 21539391]
- (30). Schelté P; Boeckler C; Frisch B; Schuber F Differential Reactivity of Maleimide and Bromoacetyl Functions with Thiols: Application to the Preparation of Liposomal Diepitope Constructs. *Bioconjug. Chem* 2000, 11 (1), 118–123. [PubMed: 10639094]
- (31). Patterson DM; Nazarova LA; Prescher JA Finding the Right (Bioorthogonal) Chemistry. *ACS Chemical Biology* 2014, 9 (3), 592–605. [PubMed: 24437719]
- (32). Faulón Marruecos D; Kienle DF; Kaar JL; Schwartz DK Grafting Density Impacts Local Nanoscale Hydrophobicity in Poly(Ethylene Glycol) Brushes. *ACS Macro Lett.* 2018, 7 (4), 498–503.
- (33). Walder R; Nelson N; Schwartz DK Super-Resolution Surface Mapping Using the Trajectories of Molecular Probes. *Nat. Commun* 2011, 2 (1), 1–7.
- (34). Mazal H; Haran G Single-Molecule FRET Methods to Study the Dynamics of Proteins at Work. *Current Opinion in Biomedical Engineering* 2019, 12, 8–17. [PubMed: 31989063]
- (35). Bednar RM; Jana S; Kuppa S; Franklin R; Beckman J; Antony E; Cooley RB; Mehl RA Genetic Incorporation of Two Mutually Orthogonal Bioorthogonal Amino Acids That Enable Efficient Protein Dual-Labeling in Cells. 2021. [bioRxiv.org](https://doi.org/10.1101/2021.04.12.439361) e-Print archive. 10.1101/2021.04.12.439361v1
- (36). Skaug MJ; Mabry J; Schwartz DK Intermittent Molecular Hopping at the Solid-Liquid Interface. *Phys. Rev. Lett* 2013, 110 (25), 256101. [PubMed: 23829747]
- (37). Yu C; Guan J; Chen K; Bae SC; Granick S Single-Molecule Observation of Long Jumps in Polymer Adsorption. *ACS Nano* 2013, 7 (11), 9735–9742. [PubMed: 24168181]
- (38). Wang D; Wu H; Schwartz DK Three-Dimensional Tracking of Interfacial Hopping Diffusion. *Phys. Rev. Lett* 2017, 119 (26), 268001. [PubMed: 29328686]
- (39). Wang D; Schwartz DK Non-Brownian Interfacial Diffusion: Flying, Hopping, and Crawling. *J. Phys. Chem. C* 2020, 124 (37), 19880–19891.
- (40). Traeger JC; Lamberty Z; Schwartz DK Influence of Oligonucleotide Grafting Density on Surface-Mediated DNA Transport and Hybridization. *ACS Nano* 2019, 13 (7), 7850–7859. [PubMed: 31244029]
- (41). Baldwin AD; Kiick KL Tunable Degradation of Maleimide-Thiol Adducts in Reducing Environments. *Bioconjug. Chem* 2011, 22 (10), 1946–1953. [PubMed: 21863904]
- (42). Alley SC; Benjamin DR; Jeffrey SC; Okeley NM; Meyer DL; Sanderson RJ; Senter PD Contribution of Linker Stability to the Activities of Anticancer Immunoconjugates. *Bioconjug. Chem* 2008, 19 (3), 759–765. [PubMed: 18314937]
- (43). Kienle DF; Falatach RM; Kaar JL; Schwartz DK Correlating Structural and Functional Heterogeneity of Immobilized Enzymes. *ACS Nano* 2018, 12 (8), 8091–8103. [PubMed: 30067333]
- (44). Betancor L; López-Gallego F; Hidalgo A; Alonso-Morales N; Dellamora-Ortiz G; CesarMateo GDO; Fernández-Lafuente R; Guisán JM Different Mechanisms of Protein Immobilization on Glutaraldehyde Activated Supports: Effect of Support Activation and Immobilization Conditions. *Enzyme Microb. Technol* 2006, 39 (4), 877–882.
- (45). Mateo C; Torres R; Fernández-Lorente G; Ortiz C; Fuentes M; Hidalgo A; López-Gallego F; Abian O; Palomo JM; Betancor L; Pessela BCC; Guisan JM; Fernández-Lafuente R Epoxy-Amino Groups: A New Tool for Improved Immobilization of Proteins by the Epoxy Method. *Biomacromolecules* 2003, 4 (3), 772–777. [PubMed: 12741797]

- (46). Zaak H; Sassi M; Fernandez-Lafuente R A New Heterofunctional Amino-Vinyl Sulfone Support to Immobilize Enzymes: Application to the Stabilization of β -Galactosidase from *Aspergillus Oryzae*. *Process Biochem.* 2018, 64, 200–205.
- (47). Monserud JH; Schwartz DK Mechanisms of Surface-Mediated DNA Hybridization. *ACS Nano* 2014, 8 (5), 4488–4499. [PubMed: 24708278]
- (48). Seitchik JL; Peeler JC; Taylor MT; Blackman ML; Rhoads TW; Cooley RB; Refakis C; Fox JM; Mehl RA Genetically Encoded Tetrazine Amino Acid Directs Rapid Site-Specific in Vivo Bioorthogonal Ligation with Trans-Cyclooctenes. *J. Am. Chem. Soc* 2012, 134 (6), 2898–2901. [PubMed: 22283158]
- (49). Chaparro Sosa AF; Kienle DF; Falatach RM; Flanagan J; Kaar JL; Schwartz DK Stabilization of Immobilized Enzymes via the Chaperone-Like Activity of Mixed Lipid Bilayers. *ACS Appl. Mater. Interfaces* 2018, 10 (23), 19504–19513. [PubMed: 29767959]
- (50). Studier FW Protein Production by Auto-Induction in High Density Shaking Cultures. *Protein Expr. Purif* 2005, 41 (1), 207–234. [PubMed: 15915565]
- (51). Aronsson G; Martensson LG; Carlsson U; Jonsson BH Folding and Stability of the N-Terminus of Human Carbonic Anhydrase II. *Biochemistry* 1995, 34 (7), 2153–2162. [PubMed: 7857926]
- (52). Gupta P; Mahlawat P; Deep S Effect of Disease-Linked Mutations on the Structure, Function, Stability and Aggregation of Human Carbonic Anhydrase II. *Int. J. Biol. Macromol* 2020, 143, 472–482. [PubMed: 31778702]

Figure 1.

Characterization of CA constructs upon immobilization on functionalized silica microparticles. A) Schematic of the immobilization of CA constructs at position 126 via AzF-DBCO (slow), Cys-maleimide (intermediate) and tetrazine-sTCO (fast) coupling chemistries. B) Specific activity of CA immobilized on DBCO, maleimide, and STCO functionalized microparticles relative to the specific activity of the soluble form of each CA construct at room temperature. C) Apparent folded fractions of immobilized CA from tryptophan fluorescence measurements, which were calculated from standard curves

of urea denaturation using soluble CA. Error bars represent the standard deviation from three independent measurements. The gradient below the plot indicates increasing ligation reaction rate constant.

Figure 2.

Representative SM-FRET trajectories of CA on functionalized silica surfaces during immobilization experiments. (A-C) x and y position as a function of time relative to the position of the final frame of the trajectory (indicated by the dashed line). The grey-shaded intervals represent periods where CA was immobilized to the surface (i.e., after ligation). (D-F) Corresponding time-dependent donor-acceptor intensity plots for the molecules in panels A-C, segmented based on folding state (red or green shaded background). (G-I) Two-dimensional plots for the trajectories above each panel. Positions have been colored to show folded (red) and unfolded (green) states. Additionally, open and filled circles represent searching states and immobilized states, respectively. The asterisk (*) indicates the position at the first frame of the trajectory.

Figure 3.

Two-dimensional surface trajectories of CA constructs during immobilization on (A) DBCO (slow), (B) maleimide (intermediate), and (C) sTCO (fast) functionalized surfaces. Trajectories (between 25–30 frames long) were randomly selected from molecules that were initially folded and immobilized at some point during the trajectory. The trajectories were segmented based on the periods during which the molecules were folded (black) and unfolded (red).

Figure 4.

Euclidian distance to immobilization for CA constructs on DBCO (blue squares), maleimide (red diamonds), and sTCO (black circles) functionalized surfaces, corresponding to slow, intermediate, and fast ligation reaction rate constants, respectively. A) Probability density distributions of the distance to immobilization. The solid lines represent the fitting function associated with a log-normal mixture model. B) Complementary cumulative distribution of the distance to immobilization. C) Folded fraction at the initial point of immobilization as a function of the distance to immobilization. All molecules included in this analysis were

initially folded upon surface adsorption. Error bars represent a 68% confidence interval of each data point obtained from 100 sub-samples of the data using a bootstrap method with replacement.