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Enhanced electrostatic discrimination of proteins on nanoparticle-coated surfaces†

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Two β-lactoglobulin (BLG) isoforms, BLGA and BLGB, were used as a test bed for the differentiation of proteins using electrostatics. In these studies, the BLGA and BLGB binding to a highly charged, cationic gold nanoparticle (GNP) modified surface was investigated by atomic force microscopy (AFM) and surface plasmon resonance (SPR) spectroscopy. The binding affinity, and more importantly, the selectivity of this surface towards these two almost identical protein isoforms were both significantly increased on the cationic GNP surface array relative to the values measured with the same free cationic GNP in solution. While protein recognition is traditionally achieved almost exclusively via orientation dependent short-range interactions such as hydrogen bonds and hydrophobic interactions, our results show the potential of protein recognition platforms based on enhanced electrostatic interactions.

To provide a challenging test bed for protein discrimination, we chose β-lactoglobulin (BLG) as a model target. BLG is a small protein of 162 amino acids and a molecular mass of ~18.4 kDa. The two most common genetic variants of this protein are BLGA and BLGB (pI = 5.2 and 5.3, respectively) that differ only in the replacement of Val 118 and Asp 64 (BLGA), by Ala 118 and Gly 64 (BLGB). The association behavior of BLGA/B has been extensively studied by various techniques and depends on factors including pH, ionic strength, and temperature. In the intermediate pH range, (pH 5–6) BLGA/B exist primarily as dimers, with a somewhat higher dimerization constant for BLGB relative to BLGA, presumably because of the location of the charged Asp 64 close to the dimer binding site in the latter.

In previous work, we have shown that 2 nm gold nanoparticles (GNP) modified with a cationic ligand, namely tetra(ethylene glycol) trimethylammonium (TTMA), can be used to discriminate between β-lactoglobulin isoforms in solution through electrostatics alone, as confirmed by the strong ionic strength dependence of the binding. Recent calculations have shown that charged patches on the protein surface, particularly at the protein dimer interface, are involved in electrostatic binding. Our hypothesis is that by fabricating a closely packed array of surface-immobilized cationic NPs, we would be able to create “hot spots” of increased local electrostatic potential formed as a result of overlap from adjacent NPs in the array. These “hot spots” would be expected to enhance electrostatic interactions with the BLG negative charge patches and therefore provide better affinity and selectivity between the two very similar isoforms without complicate modification of surface by some recognition elements. We report here the use of surface plasmon resonance spectroscopy (SPR) to demonstrate

Introduction

Selective protein recognition involves a geometric compatibility of surfaces that allows for complementary groups to engage in orientation dependent short-range interactions (e.g. hydrophobic interactions). Long-range electrostatic interactions are generally viewed as playing only a secondary role, steering the two partners into the required proximity. The level to which short-range forces are held exclusively responsible for high affinity and selectivity tends to decrease as the role of specificity diminishes. One viewpoint is that electrostatics alone cannot play the same role in recognition as orientation dependent short-range forces (e.g. hydrogen bonds and dipole-dipole interactions); however there are indications that electrostatic forces can provide a significant level of selectivity, as demonstrated in ion-exchange chromatography, ion-membrane ultrafiltration, and polyelectrolyte-induced phase separation.

In recent years, nanoparticles (NP) modified with charged ligands were successfully used to achieve selective electrostatic-based interactions with proteins in solution, mainly due to their high charge density. Application of these particles to materials applications including biosensors involves immobilization of these particles on surfaces. In this study we explored the effect of particle immobilization on protein binding affinity and selectivity.

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† Electronic supplementary information (ESI) available: Additional details of the dynamic light scattering measurements are given. See DOI: 10.1039/c3tb20377h
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both enhanced BLG affinity and isoform discrimination capability of TTMA NP-functionalized surfaces in solution.

**Experimental**

**Materials**

BLGA and BLGB were purchased from Sigma-Aldrich. Gold nanoparticles (NP) were prepared and characterized as described previously. The TTMA NP core size was 2 nm, the overall $d_\text{h}$ was 10 nm, and $\xi = 27$ mV. Sodium 3-mercaptopropylsulfonate, sodium chloride and sodium phosphate (monobasic) were purchased from Fisher Scientific. Milli-Q water was used through all experiments.

**Dynamic light scattering (DLS)**

DLS was carried out at 25 °C with a Malvern Zetasizer Nano ZS instrument equipped with temperature control and using a 633 nm He–Ne laser for backscattering at 173°. The measurement duration was 10 seconds, and 11 measurements were averaged for analysis in pH 5.5, 5 mM phosphate buffer. The distributions of the mean apparent translational diffusion coefficients ($D_T$) were determined by fitting the DLS autocorrelation functions using nonnegative constrained least squares (NNLS). The distribution of apparent diameters $D_h$ was obtained from the distribution of mean apparent translational diffusion coefficients ($D_T$) via eqn (S1).† The protein solution was adjusted to the desired pH by NaOH and filtered into a 1 mL low volume cuvette using Whatman 0.22 μm filter.

**Surface plasmon resonance (SPR) sensing experiments**

**Sensor chip fabrication.** SPR experiments were conducted using self-made sensor prepared on 0.3 mm thick borosilicate glass D263 (purchased from Howard Glass, Worcester, MA). Before coating with gold, the glass slides were sonicated in ethanol, acetone, and VERSA clean solution respectively. Before coating with gold, the glass slides were thoroughly rinsed with DI water and dried with nitrogen. A 2 nm Cr adhesion layer was first coated followed by 45 nm layer at a rate of 0.5 nm s$^{-1}$ by electron beam deposition. The coated slides were cut to 9 × 9 mm pieces. Before further modification, Au chips were sonicated in Piranha solution ($H_2O_2 : H_2SO_4 (30 : 70)$, caution! Piranha is a vigorous oxidant and should be used with extreme caution) for 5 min and the Au chip was left in Piranha for 1 hour. After rinsing with DI water, the chip was put into sodium 3-mercaptopropylsulfonate solution (10 mM, dissolved in 20 : 1 ethanol–water mixture) for 6 hours. The chip was then rinsed with DI water, dried with nitrogen and mounted to a Biacore sensor holder using a double sided tape.

**SPR experiments**

SPR analysis was performed by using the Biacore 3000 instrument. 0.001 g L$^{-1}$ TTMA-NP solution was first flowed into the SPR cell for 20 min followed by rinsing with pure buffer (pH 5.5, 5 mM phosphate) at 25 °C. Before protein injection, the TTMA-NP adsorption, as reflected by SPR signal increase, was checked to be very consistent (<1.5% deviation) in duplicated runs since the amount of TTMA on surface is critical for protein adsorption. Proteins at different concentrations from 0.0005 to 0.01 g L$^{-1}$ were flowed in to the cell for 30 min followed by rinsing with buffer solution. The flow rate throughout the experiments was 10 μL min$^{-1}$.

**Atomic force microscopy (AFM)**

AFM images were taken on a gold-coated silicon wafer (same coating as the glass SPR sensor) in tapping mode by using a Nanoscope IV scanning probe microscopy controller (Veeco Instruments, Inc.). A standard AFM probe with nominal spring constants of 42 N m$^{-1}$ and nominal resonance frequency of 320 kHz was employed. Scan rate is 1 Hz. All images were processed using the Nanoscope 5.12r2 software (Veeco, Inc.) and flattened by using a 1st order flattening function.

**Results and discussion**

Our studies began with the preparation of densely packed TTMA functionalized surfaces as shown in Fig. 1. Gold was deposited on an optical glass slide and the surface modified with an anionic self-assembled monolayer (SAM) of sodium 3-mercaptopropylsulfonate. Positively charged TTMA was then electrostatically adsorbed on the SAM. Atomic force microscopy (AFM) measurements (Fig. 2a–d) indicate that the average roughness (rms) of the surface increased from 1.1 to 1.4 nm following the modification with TTMA-NP. As the roughness of
the electron beam deposited gold surface is not much smaller than the 2 nm NP solid core, section analysis (Fig. 2e and f) was performed to identify and characterize the distribution of NP on top of a gold ridge with a relatively flat surface. This analysis revealed adjacent peaks with a peak-to-peak separation of 11–15 nm, consistent with a densely packed surface. SPR results (Fig. 3) show sequential adsorption of TTMA-NP on the SAM, followed by BLG adsorption on the TTMA-NP/SAM. The rapid initial uptake of TTMA-NP on the SAM surface indicates a strong electrostatic interaction between the two. Based on the reported equivalence that 1000 RU = 1 ng mm⁻², and the molecular weight of TTMA-NP = 100 kDa, the terminal surface coverage of TTMA-NP is 8300 NP μm⁻² (120 nm² per NP). The average spacing between the 10 nm (ligand and core diameter) TTMA-NP is then consistent with AFM studies.

Turning to protein uptake, we noticed that the adsorption of BLG on the TTMA-NP surface was much slower than the observed for TTMA-NP on the SAM (Fig. 3), as expected due to the greater electrostatic interaction expected for the particle deposition process.

A significant observation in our initial SPR experiments was that the amount of adsorbed BLGA is about twice that of BLGB. To further quantify the binding affinity, adsorption was measured at five different concentrations and the resultant binding isotherms were plotted (Fig. 4). The binding constants ($K_b$) for protein adsorption on the TTMA-NP modified surface are shown in Table 1, and compared with the binding constants of these proteins with TTMA-NP in solution. The binding affinities are clearly enhanced on the TTMA-NP modified surface, by a factor of 8 for BLGA and a factor of 4 for BLGB, relative to the respective values measured with TTMA-NP in solution. Furthermore, the selectivity between the two different BLG isoforms ($K_{BLA}/K_{BLB}$) on the TTMA-NP surface is enhanced by a factor of 2.

To understand the origin of the enhanced protein binding affinity and selectivity on surfaces relative to in solution, we first used DelPhi electrostatic modelling to model the potential contours of the two protein isoforms as shown in Fig. 5a and b. In Fig. 5b we focus on the negative “charge patches” of BLGA and B. Such charge patches are routinely invoked to explain binding of “on the wrong side” of the pI, and depend in part on the binding substrate. They can, however, also be used in a predictive way that goes beyond visualization. In Fig. 5b, the $0.5 kT e^{-1} \sim 13$ mV potential domain extends further from the protein atomic surface for BLGA than for BLGB. Thus, the overlap of protein and TTMA potential domains of opposite charge sign is more complete for BLGA due to its two additional Asp residues. In other words, BLGA interacts with more TTMA positive charges than does BLGB due to its larger “charge patch”. Our results

Fig. 2 AFM analysis of SAM surface and TTMA-NP coated surface. (a) SAM modified gold surface; (b) TTMA-NP adsorbed surface; (c) phase image of SAM modified gold surface; (d) phase image of TTMA-NP modified surface; (e) section height analysis of 100 nm region of (a) as indicated by red arrow; (f) section height analysis of 100 nm as indicated by blue arrow. Scale bars represent 400 nm.

Fig. 3 Kinetics of adsorption of TTMA-GNP on gold surface, and kinetics of subsequent adsorption of protein on TTMA-GNP-coated surface.

Fig. 4 Binding isotherms of BLGA and BLGB dimer on TTMA-NP modified surface at pH 5.5, $I = 5$ mM at 25 °C. Curves correspond to fitting by Langmuir adsorption model fitting.
Table 1 Comparison of binding parameters on surface and in solution \(^a\)

<table>
<thead>
<tr>
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<th>(K_b^\text{A} \times 10^{-6} \text{M}^{-1})</th>
<th>(K_b^\text{B} \times 10^{-6} \text{M}^{-1})</th>
<th>(\text{Ads}_{\text{max}}^\text{A} \text{ ng mm}^{-2})</th>
<th>(\text{Ads}_{\text{max}}^\text{B} \text{ ng mm}^{-2})</th>
<th>(\Delta G^\text{A} \text{ kcal mol}^{-1})</th>
<th>(\Delta G^\text{B} \text{ kcal mol}^{-1})</th>
<th>(K_b^\text{A}/K_b^\text{B})</th>
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<tbody>
<tr>
<td>Surface (^b)</td>
<td>29.1</td>
<td>8.9</td>
<td>1.25</td>
<td>1.25</td>
<td>–10.3</td>
<td>–9.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Solution (^b)</td>
<td>3.8</td>
<td>2.1</td>
<td>NA</td>
<td>NA</td>
<td>–9.1</td>
<td>–8.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\(^a\) The \(^A\) and \(^B\) notations indicate BLGA and BLGB respectively. \(^b\) Measured by SPR (surface) and isothermal titration calorimetry (ITC – solution).

\[ K_b^\text{A} = (K_b^\text{B})^\psi_{\text{A}}/\psi_{\text{B}} \]  \(3\)

In the strongest binding case shown in Fig. 5d, BLG resides at the position of maximum potential overlap of three NP, and \(\psi_{\text{A}}/\psi_{\text{B}}\) could be as large as 3, assuming potentials are additive. A purely geometric consideration would suggest a total of 6 binding sites per TTMA-NP: two sites with \(\psi_{\text{A}}/\psi_{\text{B}} = 3\), three sites with \(\psi_{\text{A}}/\psi_{\text{B}} = 2\), and one site without potential overlap, as in Fig. 5d. However, since the average stoichiometry of binding from Fig. 4 is 2.4 BLG dimers per TTMA-NP, the Boltzmann distribution of the energy \(E \sim \gamma_x\) assures that most proteins are bound in regions of largest potential overlap \(\psi_{\text{A}}/\psi_{\text{B}} = 3\). Substituting this value into eqn (3) reveals that this ratio of potentials yields \(K_b^\text{A}/K_b^\text{B}\) far greater than the experimental values, suggesting compensating effects. First, the simple representations in Fig. 5c and d and in eqn (3) neglect the repulsive interaction between the cationic TTMA groups and the protein positive domain. Second, repulsion among the positive protein domains within the relatively planar bound layer can be significant, as observed in other adsorption studies. \(^{21,22}\) Such repulsions are reduced when binding occurs in a spherical geometry such as that of free TTMA-NP in solution (Fig. 5c), accounting for the much higher BLG:TTMA-NP stoichiometry found in this case (\(n = 27\) and 16 for BLGA and B respectively). \(^9\) Regarding the selectivity, \(K_b^\text{A}/K_b^\text{B}\) is logarithmically proportional to \(\Delta G^\text{A} \sim \Delta G^\text{B}\), therefore the uniform increase, by a factor of 1.5, of \(\Delta G\) in the case of BLG binding to the TTMA-NP monolayer leads directly to the observed two-fold enhancement in \(K_b^\text{A}/K_b^\text{B}\).

Conclusions

A closely packed array of cationic NP was used to determine the effects of NP adsorption on electrostatic protein binding affinity and selectivity. The association of BLG on the TTMA-NP based surface is substantially stronger than with TTMA-NP in solution due to the enhanced electrostatic interactions between the overlapping positive potentials of adjacent TTMA-NP in the array with negative charge patches on the protein surface. Significantly, selectivity increased along with affinity: a \(\geq 3\)-fold difference in affinity was observed between BLGA and BLGB that differ only by 2 amino acids. This study presents the possibility of engineering surfaces for enhanced selectivity and perhaps even specificity using electrostatics, an outcome of importance for sensing and separations applications.
Acknowledgements

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Notes and references