Electronic Supplementary Information

Competitive multicomponent anion exchange adsorption of proteins at the single molecule level

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Table S1. Ensemble kinetic fitting results of desorption times. The values reported here are for the data shown in Figure 1 and Figure S1.

[Insulin] (nM)	0	3	30	300	3000
Weighted average τ (s)	38	31	39	47	39
Amplitude τ_1	83 ± 2 %	81 ± 2 %	82 ± 1 %	76 ± 1 %	76 ± 8 %
τ_1 (ms)	20.2 ± 0.6	17.6 ± 0.7	21.6 ± 0.5	24.1 ± 0.9	21 ± 5
Amplitude τ_2	16.0 ± 0.8 %	16 ± 1 %	15.6 ± 0.8 %	22 ± 1 %	20 ± 10 %
τ_2	89 ± 4	63 ± 3	87 ± 4	91 ± 5	60 ± 20
Amplitude τ_3	1.5 ± 0.2 %	2.1 ± 0.2	2.0 ± 0.2 %	2.2 ± 0.5 %	2.5 ± 0.9 %
τ_3	490 ± 40	300 ± 20	410 ± 30	380 ± 40	400 ± 100

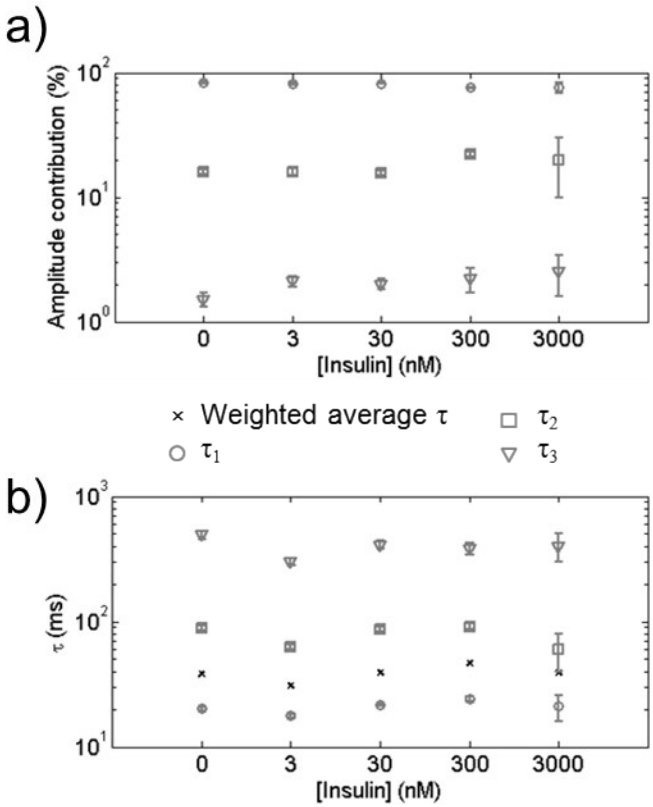

Figure S1. The (a) amplitude and (b) lifetime components of the Figure 1fitting results, which are also reported in Table S1.

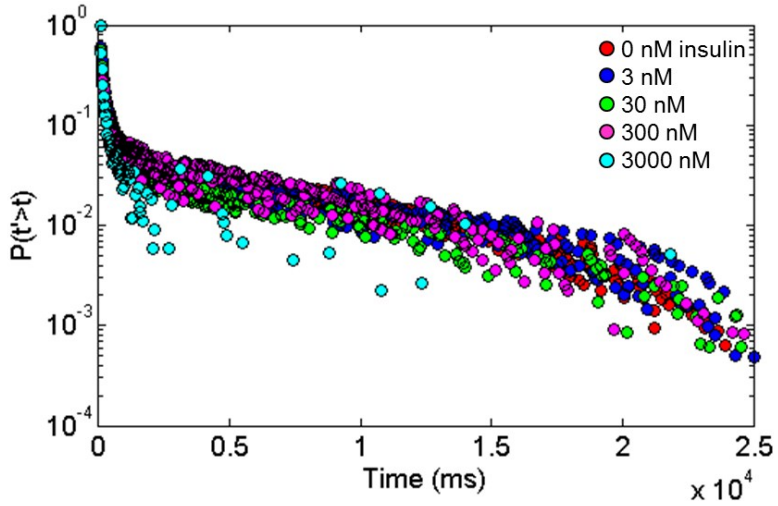

Figure S2. Adsorption kinetics of α-lactalbumin are unchanged in the presence of insulin. Cumulative distributions of the α-lactalbumin waiting time between binding events at individual ligands as a function of competitive insulin concentration for all spermine ligands in a 30 x 30 μm^2 area. The data represents the mean from five replicate measurements.

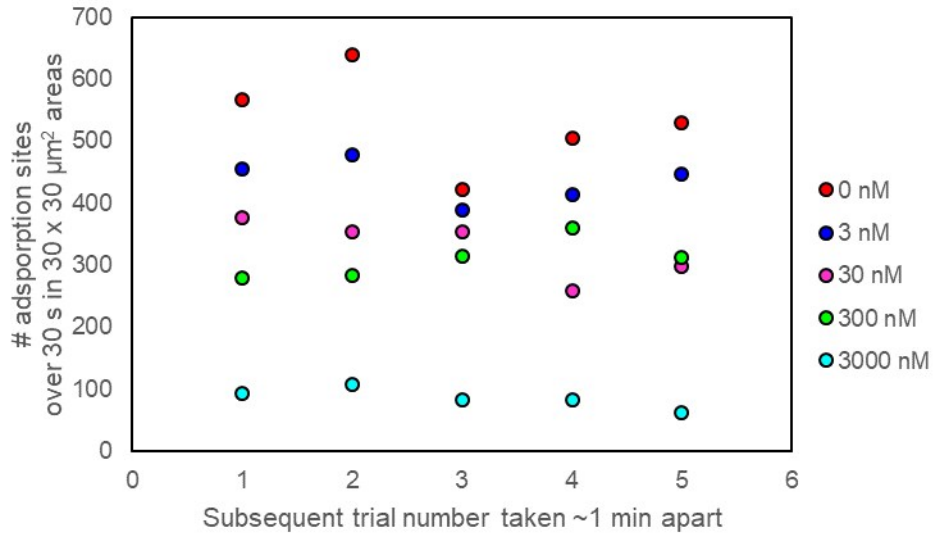

Figure S3. The number of identified adsorption sites does not vary with time. The sample was allowed to equilibrate for 10 min after first introducing the analyte/competitor solution and five distinct trials (1,000 frames, 33 Hz frame rate, 30 s total) were measured ~ 1 min apart. For all insulin concentrations, the number of identified adsorption sites is randomly distributed and no systematic increase or decrease is observed over time, suggesting that our measurements are made under equilibrium conditions.

METHODS

Fluorescent dye labeling of α-lactalbumin

The analyte α-lactalbumin (Sigma Aldrich stock no. L6010) was labeled with Alexa 555 as previously described.[1] The labeled protein was purified using DEAE Sepharose FF (GE Healthcare Bio-Sciences, Uppsala, Sweden) packed in a glass column (41 mm height × 5 mm inner diameter) to separate single-fluorophore-labeled from unlabeled protein. The column initially was equilibrated with 10 column volumes (CV) of 25 mM Tris, pH 8.0 ("Buffer A"). Alexa 555-labeled α-lactalbumin was then loaded onto the column, followed by subsequent washing with 5 CV of buffer A and elution over 40 CV with a linear gradient from Buffer A to Buffer A + 1 M NaCl. Fractions with an estimated fluorophore-to-protein ratio of 1.0 were pooled and analyzed using MALDI-TOF to confirm the presence of only single-fluor labeled protein (Figure S4). MALDI-TOF mass spectrometry of α-lactalbumin was performed on an AB Sciex 4800 MALDI-TOF/TOF mass spectrometer with AB Sciex 4000 Series Data Explorer control and processing software (V3.7.1 Build 1, AB Sciex). Sample preparation was as described previously[2] without any proteolytic digestion and calibration peptides.. The MALDI-TOF spectra obtained for Alexa α-lactalbumin after chromatographic purification confirmed the presence of singly-labeled protein when compared with spectra for unlabeled α-lactalbumin (Figure S4).

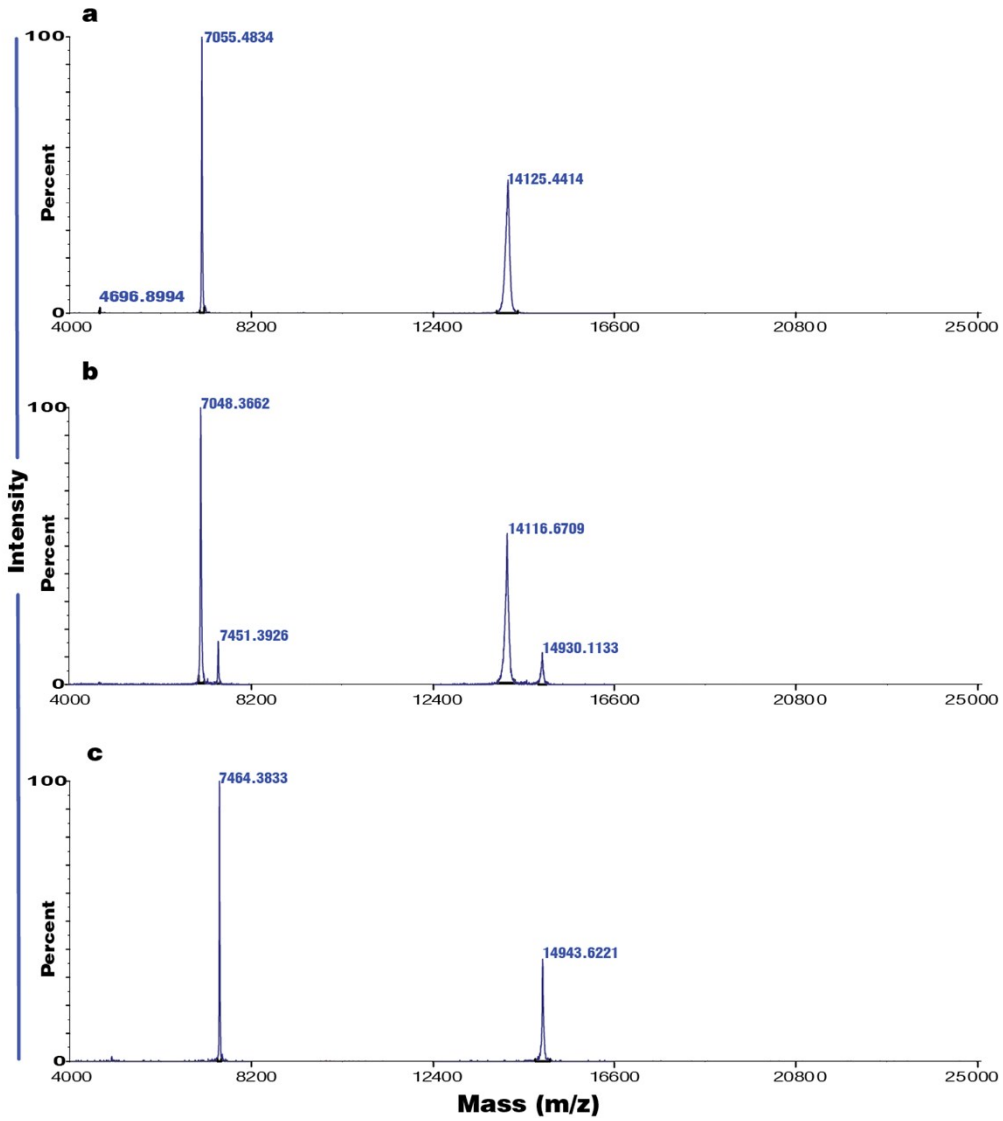

Figure S4. Comparison of MALDI-TOF spectra obtained for (a) unlabeled α-lactalbumin, (b) Alexa 555-labeled α-lactalbumin prior to the final anion exchange purification step and (c) Alexa 555-labeled α-lactalbumin after the final anion exchange purification step (fractions with an estimated fluorophore-to-protein ratio of 1.0 were pooled before MALDI-TOF analysis). The peak at ~14,100 corresponds to unlabeled α-lactalbumin and ~14,900 corresponds to singly Alexa 555-labeled α-lactalbumin.

Agarose surface preparation for single molecule spectroscopy studies

Glass coverslips (No. 1; VWR, 22 x 22 mm) were cleaned with isopropyl alcohol then cleaned with oxygen plasma for 2 min. Silicon templates (Grace BioLabs, Bend, OR) were attached to the coverslips, and 1 mL of hot (> 65 °C) 1% (w/w) agarose solution (Type I, low

EEO; Sigma-Aldrich, St. Louis, MO) was spun onto each coverslip at 3000 rpm using a Brewer Science Cee 200CBX precision coat-bake system. Spermine-activated agarose surfaces were prepared as discussed previously[1] with some modifications. Briefly, spermine ligands were attached using aldehyde functionalities introduced to the agarose surfaces by 18 hours treatment with 20 mM $NaIO_4$. After washing with deionized water, the activated surfaces were treated with a 250 μM solution of spermine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) in coupling buffer (100 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.2) in the presence of 4.6 M $NaBH_3CN$ (Thermo Scientific AminoLink Reductant). Excess uncoupled spermine was removed by rinsing gently with coupling buffer, and unreacted aldehyde sites were reduced with 66 mM of $NaBH_4$ (in 25% ethanol/75% phosphate-buffered saline) for 5 min. The surfaces were finally rinsed gently with coupling buffer and were immersed in 6.0 mL coupling buffer for 24 h to leach out any excess uncoupled spermine. The entire procedure was carried out at room temperature. Control agarose surfaces were prepared following the procedure outlined above, but without the spermine in coupling buffer. After completion of the entire procedure, a custom flow chamber (Grace BioLabs) was placed over the agarose thin film, and 500 pM Alexa 555 labeled α-lactalbumin and 3, 30, 300, or 3000 nM insulin (Sigma) in 5 mM Tris, pH 8 was flowed at 5 μL/min using a Genie Plus flow system (Kent Scientific). Samples were allowed to equilibrate for 10 min once the protein solution was flowed over the stationary phase.

Measurement of spermine density

^{14}C-labeled spermine tetrahydrochloride (American Radiolabeled Chemicals, Inc, St. Louis, MO) was used for estimating the (very low) ligand density of the adsorbents. Fifty μL of 250 μM ^{14}C-spermine (12.5 nmole) were applied on the periodate-activated agarose film and

after surface modification following the same procedure for "cold" spermine as described above, the ^{14}C –spermine-coated coverslips were immersed into 1 mL of sodium hypochlorite solution for 45 min at 65 $^{\circ}C$ to dissolve the agarose film. Then 10 mL of Hionic-Fluor™ (PerkinElmer) were added to the samples. Measurement of the radiolabeled spermine was performed on a Beckman LS 6500 Liquid Scintillation Counter in automatic count mode. The coupling efficiency (CE%) was estimated as the ratio of the Observed Standard Count Rate (cpm) of the sample containing the dissolved ^{14}C-spermine agarose to the Observed Standard Count Rate (cpm) of the initial amount of stock ^{14}C-spermine that was applied onto the agarose film (adjusted for dilution). Control experiments confirmed that the sodium hypochlorite solution did not affect counting efficiency. Typical values of CE were around 0.02%. The immobilized spermine concentration was then estimated at 0.49 mM given that the volume of the agarose thin film on the coverslip was π x 15/2 mm x 5/2 mm x 88 nm = 5.1 x 10^{-3} mm^3.

Single molecule spectroscopy measurements and analysis

A home-built wide field total internal reflection fluorescence microscope was used as previously described.[3] Briefly, a 532 nm solid state laser (Coherent, Compass 315M-100SL) was reflected onto the edge of a high-numerical aperture oil-immersion objective (Carl-Zeiss, alpha Plan-Fluar, 1.45 NA, 100x). The evanescent wave excited the sample at a penetration depth of ~85 nm and intensity of ~5 mW/cm^2. The emission was collected in an epi-fluorescent configuration and detected on an EMCCD (Andor, iXon 897) at an electron multiplying gain of 300, integration time of 30 ms, and frame rate of 33 Hz. Five subsequent trials of 1,000 frames each were taken ~1 min after one another for the same area of the same sample for each solution.

Data was analyzed as previously reported by motion-blur points accumulation for imaging in nanoscale topography (mbPAINT) using MATLAB 2014b.[4] The emission of diffusive unbound proteins is not observable due to motion blur, while the diffraction-limited point spread functions of stochastically adsorbed proteins to the surface are observed and the centroid positions are super-localized. Repetitive binding to the same ligand locations leads to a super-resolution image at ~30 nm. Binding kinetics were calculated from counting the number and length of events from frame to frame.

References

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(2) Mitchell, M.; Mali, S.; King, C. C.; Bark, S. J. Enhancing MALDI Time-of-Flight Mass Spectrometer Performance through Spectrum Averaging. *PLoS One* **2015**, *10*, e0120932.

(3) Kisley, L.; Chang, W.-S.; Cooper, D.; Mansur, A. P.; Landes, C. F. Extending Single Molecule Fluorescence Observation Time by Amplitude-Modulated Excitation. *Methods Appl. Fluoresc.* **2013**, *1*, 037001–37001.

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