Development of Escherichia coli Asparaginase II for Immunosensing: A Trade-Off between Receptor Density and Sensing Efficiency

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ABSTRACT: The clinical success of Escherichia coli l-asparaginase II (EcAII) as a front line chemotherapeutic agent for acute lymphoblastic leukemia (ALL) is often compromised because of its silent inactivation by neutralizing antibodies. Timely detection of silent immune response can rely on immobilizing EcAII, to capture and detect anti-EcAII antibodies. Having recently reported the use of a portable surface plasmon resonance (SPR) sensing device to detect anti-EcAII antibodies in undiluted serum from children undergoing therapy for ALL (Aubé et al., ACS Sensors 2016, 1 (11), 1358–1365), here we investigate the impact of the quaternary structure and the mode of immobilization of EcAII onto low-fouling SPR sensor chips on the sensitivity and reproducibility of immunosensing. We show that the native tetrameric structure of EcAII, while being essential for activity, is not required for antibody recognition because monomeric EcAII is equally antigenic. By modulating the mode of immobilization, we observed that low-density surface coverage obtained upon covalent immobilization allowed each tetrameric EcAII to bind up to two antibody molecules, whereas high-density surface coverage arising from metal chelation by N- or C-terminal histidine-tag reduced the sensing efficiency to less than one antibody molecule per tetramer. Nonetheless, immobilization of EcAII by metal chelation procured up to 10-fold greater surface coverage, thus resulting in increased SPR sensitivity and allowing reliable detection of lower analyte concentrations. Importantly, only metal chelation achieved highly reproducible immobilization of EcAII, providing the sensing reproducibility that is required for plasmonic sensing in clinical samples. This report sheds light on the impact of multiple factors that need to be considered to optimize the practical applications of plasmonic sensors.

INTRODUCTION

The E. coli l-asparaginase II (EcAII) isozyme hydrolyses L-Asn into L-Asp with a high catalytic efficiency. It is a critical component of chemotherapy for childhood acute lymphoblastic leukemia (ALL) and has been in the World Health Organization’s list of essential medicines since 1995.1–3 However, its use may be compromised by allergic reactions, overt or silent.4–6 The main concern relative to the silent hypersensitivity that occurs in 5–46% of patients is the development of neutralizing antibodies that result in silent inactivation of EcAII, thus reducing treatment efficacy.4–6 As a counterpart to its therapeutic use, EcAII is also used to capture and thus detect anti-EcAII antibodies in patients.12,13 The crystal structure of native EcAII has been resolved in different space groups, free or complexed, and for several mutants, revealing a highly packed homotetrameric structure exhibiting four identical active sites formed by complementation of the so-called intimate homodimers.1,14 Several antigenic determinants of EcAII have been identified, including a dominant B-cell conformational epitope. However, little is known about the antigenicity of EcAII in an immobilized form, which is an essential aspect for immunosensing purposes.15

We recently reported the application of a portable immunosensing device based on surface plasmon resonance (SPR) to detect anti-EcAII antibodies in undiluted serum from children undergoing therapy for ALL (Aubé et al., 2016). We strive to work directly with complex biological media to reduce the impact that the sample pretreatment may have on the analyte and to reduce the time of analysis. Several challenges were encountered during that study, the principal of which was poor and/or irreproducible surface immobilization of the native EcAII antigen. Here, we report a detailed examination of the mode of presentation of EcAII on SPR sensor chips to identify the immobilization chemistry eliciting optimal immunosensing.
properties.\textsuperscript{12,16–20} Indeed, the mode of surface immobilization may preclude efficient antibody recognition if the binding site (epitope) or the surrounding regions are partially masked in the ensemble of EcAII molecules.\textsuperscript{7,25} Furthermore, alterations in the quaternary structure upon immobilization could affect its antigenicity, where conformation or subunit assembly is essential, reducing the SPR response.

To this effect, we compared heterogeneous surface immobilization of native EcAII by covalent cross-linking via its surface-exposed lysine residues and homogeneous surface immobilization by coordination of N- or C-terminal histidine (His)-tags. The quaternary structure, the activity, and the antigenicity of native EcAII and EcAII bearing N- or C-terminal 6-His-tags were compared before and after surface immobilization. We then validated those results in the context of SPR immunosensing in serum. This analytical method has many advantages over the enzyme-linked immunosorbent assay (ELISA) that is commonly used for the clinical monitoring of anti-EcAII antibodies. Indeed, SPR immunosensing can offer real-time, label-free, and on-site detection and quantification of antibodies. Using low-fouling self-assembled monolayer (SAM) surface technology to reduce nonspecific interactions, we assessed the sensitivity of antibody sensing by SPR in undiluted serum.\textsuperscript{22} Changes to the quaternary structure had little influence on the receptor antigenicity. Although the His-tagged EcAII displayed lower sensing efficiency than the native EcAII, it provided increased surface coverage and reproducibility of immobilization, ultimately procuring significantly increased immunodetection sensitivity. These results shed light on the challenges encountered in our recently reported detection of serum anti-asparaginase antibodies in the sera of children undergoing chemotherapy\textsuperscript{23} and the challenges expected to be encountered by others developing sensors to monitor the immunogenic response of patients undergoing therapy with biologic-type drugs.

\section*{RESULTS AND DISCUSSION}

EcAII functions as a tetramer, where the intimate homodimers further dimerize to form the functional homotetramer.\textsuperscript{1,24–28} Among the many characterized linear T- and B-cell epitopes, a dominant conformational B-cell epitope has been identified on EcAII. This conformational epitope, present four times on EcAII, is formed by four different immunogenic segments clustered around the entrance of each of the four identical active sites and may be the principal target for neutralizing antibodies and silent inactivation\textsuperscript{15,25–31} (Figure 1A,B).

Toward the goal of capturing anti-EcAII antibodies, the EcAII protein was surface-immobilized to act as a receptor. Its numerous surface-exposed lysine residues (approximately 76) make it possible to undertake covalent, randomly oriented immobilization onto gold-coated SPR sensor chips. In parallel, we developed several His-tagged EcAII variants to allow oriented immobilization by coordination with surface-immobilized nitrotriacetic acid (NTA)-cobalt (Co)-functionalized antifouling peptides (Figure 1C).

In addition to its functional tetrameric form, EcAII forms alternative oligomeric states in solution as a function of protein preparation and storage conditions.\textsuperscript{8,20} Commercial preparations may contain up to 20% monomer and higher multimerization states (octamer and dodecamer, among others) that are less active than the tetramer.\textsuperscript{8} Higher-state oligomers may present different antigenic determinants. Because modifying the protein sequence may alter the oligomerization state and thus alter immunogenicity, we investigated the quaternary structure of the His-tagged forms of EcAII.

Recombinant N- and C-terminally His-tagged forms of EcAII (N21-, N26- and C8-EcAII) were named after the length of the fused tags that added 21 and 26 residues at the N-terminus or 8 residues at the C-terminus (Figures 1C and S1). Replacement of the N-terminal signal peptide by the N-terminal His-tags led to lower yields of soluble proteins (9–15 and 1–16 mg L\textsuperscript{−1} of N21- and N26-EcAII culture, respectively) relative to the periplasmic overexpression of C8-EcAII (60–80 mg L\textsuperscript{−1} of culture), despite the overall expression levels being similar as observed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (results not shown). This is
consistent with the expected toxicity of cytosolic EcAII, in accordance with the 3 orders of magnitude difference in affinity for L-Asn between the constitutive cytosolic EcAII ($K_M = 3.5 \text{ mM}$) and the periplasmic EcAII ($K_M = 10 \mu \text{ M}$) isoforms. Each His-tagged EcAII was purified to $\geq 90\%$ homogeneity, mostly in the tetrameric form (Figure 2). Accurate mass determination using liquid chromatography–mass spectrometry (LC–MS) confirmed the expected molecular weight (MW) for the commercial Kidrolase (native EcAII) and for the processed C8-EcAII (native signal peptide cleaved) and revealed processing of the N-terminal methionine for both N21-EcAII (black), N26-EcAII (blue), C8-EcAII (red), and monomeric C8-EcAII (red dashes). (C) Size exclusion chromatograms at 0.5 mg mL$^{-1}$ (inset at 0.1 mg mL$^{-1}$) under the conditions used for surface immobilization. (D) SDS-PAGE of Kidrolase (1), N21-EcAII (2), N26-EcAII (3), and C8-EcAII (4). Left: broad range MW marker (in kDa). (E) BN-PAGE, (a); light blue native-PAGE (LBN-PAGE, b); and CN-PAGE for Kidrolase (1), N21-EcAII (2), N26-EcAII (3), C8-EcAII (4), and monomeric C8-EcAII (5) using 25 µg (c) or 12.5 µg (d) of each protein. Proteins were stored at $-80 \degree C$ with or without glycerol (GOH) and prepared with or without 6-aminocaproic acid (6-ACA) in the loading dye. Aggregates (A) are indicated for N21-EcAII.

| protein | conformational stability | antigenicity |
|---------|--------------------------|-------------|
|         | specific activity (U mg$^{-1}$) | $T_m$ (°C) | $K_{D(catalysis)}$ (mg mL$^{-1}$) | $K_{D(B-NTA)}$ (mg mL$^{-1}$) |
| Kidrolase | 118 ± 4.3 | 60.8 ± 0.9 | 210 ± 13 | n.a.$^e$ |
| N21-EcAII | 27.5 ± 0.5 | 60.5 ± 0.3 | 120 ± 7 | 290 ± 19 |
| N26-EcAII | 26.6 ± 0.6 | 59.8 ± 0.2 | 160 ± 9 | 310 ± 22 |
| C8-EcAII | 44.7 ± 1.5 | n.d.$^d$ | 220 ± 13 | 380 ± 35 |
| C8-EcAIIIm | 0.91 ± 0.16 | n.d.$^d$ | 260 ± 15 | 390 ± 38 |

$^a$Kidrolase was freshly reconstituted; N21-EcAII, N26-EcAII, and C8-EcAIIIm were stored at $-80 \degree C$, and C8-EcAII was stored at $4 \degree C$. $^b$Not determined because of complex unfolding profile (no or more than one transition). $^c$Detection of polyclonal anti-EcAII antibodies (pAb) with revelation using conditions of intermediate sensitivity. $^d$Randomly oriented adsorption on MaxiSorp microplates. $^e$Oriented coordination of EcAII by His-tags using Ni-NTA-coated microplates. $^f$Not applicable (no His-tag).

The maintenance of native tetrameric quaternary structure might be an advantage when using EcAII as a receptor for antibody capture, according to the report of a dominant conformational B-cell epitope$^{33,34,41}$ (Figure 1). The commercial EcAII preparation (Kidrolase) was mainly tetrameric in solution according to analytical size exclusion chromatography (SEC; expected MW 138.4 kDa; observed MW $\approx 120$ kDa) (Figure 2A). It also included traces of octamer (expected MW 415 kDa; observed MW $\approx 315$ kDa) and dodecamer (expected MW 415 kDa; observed MW $\approx 456$ kDa) but no detectable monomer. The proportion of tetramer in the native EcAII decreased from 99% to 90% with a concomitant increase in the octameric form upon increasing the protein concentration (0.1–5 mg mL$^{-1}$), consistent with previous reports.$^{33,41}$
Electrophoresis under native conditions [clear native (CN)- and blue native (BN)-PAGE] further confirmed that freshly reconstituted, native EcAII occurs principally as a tetramer, with some octamer and dodecamer and traces of higher state oligomers.

As for native EcAII, freshly isolated N21-, N26-, and C8-EcAII were all predominantly tetrameric in solution (SEC, data not shown). Although N26-EcAII remained predominantly tetrameric after storage at −80 °C, consistent with its activity, N21-EcAII showed a marked increase in octamer and dodecamer and tended to aggregate over long-term storage. Storage in 15% glycerol (GOH) stabilized the tetrameric form of N21-EcAII (Figure 2C,E). The tendency of N21-EcAII to form higher-state oligomers was also observed on CN-PAGE, whereas N26-EcAII remained mainly tetrameric. By contrast, the C-terminally tagged C8-EcAII mostly dissociated into monomers after flash-freezing/thawing and did not reassociate in solution over time, consistent with its loss of activity (Figure 2B,C,E). However, C8-EcAII stored at 4 °C was mainly tetrameric according to SEC though it appeared roughly 50% dissociated into monomer on BN-PAGE. Interestingly, monomeric C8-EcAII appeared to undergo reassociation into tetramer and octamer during the course of CN-PAGE and appeared similar to freshly isolated C8-EcAII. Reassociation was not observed under any other condition, including BN-PAGE (Figure 2E). Thus, a variety of conditions maintain the monomeric form of the dissociated C8-EcAII, including the lengthy SEC at 4 °C, activity assays at 37 °C over 10 min, and BN-PAGE in the presence of Coomassie blue G-250, whereas CN-PAGE promoted full reassociation of monomeric C8-EcAII into native-like oligomeric forms. Although we did not further investigate the specific factors that promote this reassociation, we have identified conditions where each N- and C-terminally tagged EcAII is tetrameric and active upon storage and conditions where C8-EcAII is maintained in an inactive, monomeric form.

The far-ultraviolet circular dichroism (UV CD) spectra of all EcAII variants were consistent with well-folded α/β proteins (Figure S3). The minima for α-helices differed somewhat for N-terminally tagged EcAII (θ222nmmin < θ208nmmax) relative to native and C8-EcAII (θ222nmmax > θ208nmmin). This likely reflects the contribution of their N-terminal extensions but may also result from their cystolic expressions. Importantly, the CD spectrum of monomeric C8-EcAII is nearly identical to native tetrameric EcAII and tetrameric C8-EcAII, indicating that the secondary structure is maintained upon dissociating into a monomer.

Intrinsic fluorescence revealed a similar packing of aromatic residues for all tetrameric EcAII variants (λmax = 320 nm). By contrast, monomeric C8-EcAII displayed increased solvent exposure of aromatic residues (λmax = 330 nm), consistent with altered packing that may result either from subunit dissociation or from changes in the tertiary structure. The latter is supported by the lack of reassociation into a tetramer (Figures 2B,C and S4). Thermal denaturation revealed a cooperative and apparent two-state denaturation profile for native and N-terminally tagged EcAII, with Tm consistent with CD-derived Tm values reported for native EcAII and EcAII (Table 1 and Figure S4). A more complex unfolding profile was observed for tetrameric C8-EcAII (more than one transition), precluding the determination of Tm. No transition was seen for monomeric C8-EcAII (Figure S4). Upon refolding, native and N-terminally tagged EcAII recovered 75–80% of the initial fluorescence intensity. Interestingly, this was accompanied by a 7 nm red shift (λmax = 327 nm), similar to monomeric C8-EcAII before thermal denaturation (λmax = 330 nm) (Figure S5), suggesting refolding into monomers. Overall, the N-terminally His-tagged EcAII variants show association and folding properties more similar to native EcAII than the C-terminally tagged EcAII.

To verify if the quaternary structure, the His-tag at the N- or C-terminus or the immobilization mode of EcAII (random or oriented) modulate the sensing properties for detecting the anti-EcAII antibodies, antigenicity was analyzed using ELISA. Titration of polyclonal rabbit IgG was determined with the EcAII variants randomly adsorbed on a surface that binds both the hydrophilic and hydrophobic regions of proteins (MaxiSorp) or with His-tagged EcAII variants immobilized in an oriented manner via Ni-NTA coordination (Figure S6). Development was performed under conditions of high, intermediate, and low sensitivity by modulating the concentration of H2O2. In each case, the dynamic range of the binding assay spanned 2 orders of magnitude and was similar for all forms of EcAII (Figure S7 and Table S5). The apparent dissociation constant (KD) ranged from 10−26 ng mL−1 (~100–160 pM) under high sensitivity conditions, 120–260 ng mL−1 (~0.7–1.7 nM) at intermediate sensitivity, and 0.75–1.5 μg mL−1 (~5–10 nM) at low sensitivity (Tables S5 and S6). Monomeric C8-EcAII showed a slightly lower antigenicity (higher KD) than tetrameric EcAII variants; among the tetrameric variants, both N-terminally tagged EcAII showed slightly higher antigenicity (lower KD) than the native form (P = 0.0001). Antibody titration with His-tagged EcAII oriented on Ni-NTA-coated plates gave a 1.5- to 2.5-fold higher KD than for randomly oriented immobilization, indicating less efficient antibody recognition (Figure 3 and Table 1). Overall, the quaternary structure of EcAII has little influence on its antigenicity under the conditions tested (Figures 3 and S8), yet the mode of immobilization has a clearly discernable effect on antibody–antigen affinity. Furthermore, we demonstrated that the presence of a His-tag is compatible with maintaining the antigenicity of EcAII.

The impact of quaternary structure and mode of immobilization of EcAII on the extent of surface coverage was monitored on gold chips, and the immunosensing properties of immobilized EcAII variants were assessed using SPR. Native EcAII (Kidrolase) was immobilized onto the gold sensing surface in a randomly oriented fashion by covalent cross-linking of lysine residues using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide N-hydroxysuccinimide (EDC/NHS) chemistry, whereas His-tagged EcAII variants were immobilized in an oriented fashion by metal affinity coordination (Co-NTA) (Figures 4 and S9). At equal protein concentration, the protein coverage (I) determined for oriented His-tagged EcAII was 5-fold to more than 10-fold greater than for randomly oriented native EcAII (Kidrolase) (Table 2 and Figure S5). As for covalently bound Kidrolase, the metal-chelated His-tagged EcAII variants remained surface-bound upon extensive washing of the sensor chip, with the exception of the monomeric C8-EcAII that showed some dissociation (Figure 4A). Importantly, the surface coverage for His-tagged EcAII was highly reproducible with relative standard deviation (RSD) ranging between 3 and 18%, contrary to cross-linked Kidrolase that afforded poor reproducibility (RSD 50–70%). We confirmed that the glycine present in the lyophilized Kidrolase preparation was not the cause of the poor surface coverage observed because no significant difference was observed upon dialysis of
the resuspended Kidrolase ($\Gamma = 60.25 \pm 43.3$ ng cm$^{-2}$ without dialysis or $45.8 \pm 44.7$ ng cm$^{-2}$ with dialysis). This suggests that the cross-linking method itself leads to lower yields and lower reproducibility of immobilization than the metal coordination of terminal His-tags, which is an important consideration for the development of an immunosensor.

The lower surface coverage of C8-EcAII (whether monomeric or tetrameric) relative to N26-EcAII or N21-EcAII correlates with our observation of ready dissociation of C8-EcAII from the Ni-NTA purification column (with 70 mM imidazole) relative to the latter (with 250 mM imidazole). The short C-terminal tag (8 residues) may be less accessible for chelation than the 20- and 25-residue N-terminal tags. The activity of EcAII variants immobilized on the gold sensing surface was monitored to assess their integrity (Figure 4B). The same pattern of specific activity was observed as that for free EcAII, where native EcAII was 2 to 4-fold more active than the His-tagged forms. In addition, the immobilized EcAII was 2.6 to 3.6-fold more active than the free enzyme (Table 2), consistent with other reports of enzymes that display higher specific activity upon immobilization.45,46 Our results suggest that the tetrameric proteins remained essentially intact when immobilized on the sensing surface. Surface-immobilized monomeric C8-EcAII was inactive, indicating that it did not reassemble into an active tetramer upon immobilization on the sensor chip.

The greater surface density observed for both N-terminally His-tagged EcAII relative to Kidrolase and C8-EcAII suggests that the N-terminally tagged EcAII may constitute more effective receptors for the detection of the anti-EcAII antibody (Ab). This was verified by assessing the SPR immunosensing signal using the P4SPR instrument and determined as the wavelength shift upon binding of polyclonal anti-EcAII antibodies (Ab) to immobilized EcAII antigenic receptors (Ag) directly in undiluted serum. The immunodetection was performed at two antibody concentrations within an analytically relevant range (15 and 150 μg L$^{-1}$).23 At 15 μg L$^{-1}$ antibody concentration (∼100 nM), N26-EcAII and C8-EcAII provided approximately 2-fold greater SPR detection signal than did native EcAII (Kidrolase) and monomeric C8-EcAII. This difference was accentuated at 150 μg L$^{-1}$ (∼1 μM) antibody concentration, where oriented tetrameric N- and C-terminally tagged-EcAII provided approximately 5-fold and 3-fold greater detection signal than nonoriented cross-linked Kidrolase and oriented monomeric C8-EcAII, respectively.

Figure 3. Antigenicity of immobilized native and His-tagged EcAII in ELISA. Titration of rabbit polyclonal anti-EcAII antibodies (IgG) in ELISA with native EcAII (black), N21-EcAII (green), N26-EcAII (blue), and tetrameric (red) or monomeric (red, dashed line) C8-EcAII. The data were fitted to a one-site binding model. The error bars show standard deviation for triplicate reads. (A) Proteins were randomly adsorbed onto Nunc-Maxisorp plates. Detection was performed with Fc-specific goat horse radish peroxidase (HRP)-conjugated antirabbit IgG (1:1000 dilution) and 0.16% H$_2$O$_2$. (B) ELISA with His-tagged variants immobilized in an oriented manner on Ni-NTA-coated microplates.

Figure 4. Immobilization and on-chip activity of EcAII variants. Surface immobilization of EcAII variants was followed upon the injection of 40 μg of protein (0.1 mg mL$^{-1}$), and the activity of EcAII was monitored on 9 × 9 mm gold-coated glass slides for Kidrolase (black) or His-tagged EcAII: N21-EcAII (green), N26-EcAII (blue), and C8-EcAII (red). The monomeric variant is identified. (A) SPR sensograms for randomly oriented, cross-linked Kidrolase and oriented Co-NTA-coordinated His-tagged EcAII. The arrow indicates a wash step. (B) On-chip activity measurements for surface-immobilized EcAII variants monitored using the GDH-coupled assay. Functionalized chips lacking EcAII served as a blank (gold curve, partly masked). Each curve represents the average of three experiments except for monomeric C8-EcAII that is a duplicate.
Table 2. Immobilization and Immunosensing Properties of EcAII (Ag) Receptors

| protein            | \( \Delta_{\text{spr}} \) (nm) | surface density (pmol cm\(^{-2}\)) | activity\(^a\) (U mg\(^{-1}\)) | distance c.t.c. (nm) | \( \Delta_{\text{spr}} \) (nm) | sensing efficiency (Ab/Ag binding ratio) |
|--------------------|-------------------------------|-----------------------------------|----------------------------------|---------------------|-------------------------------|-----------------------------------------|
| Kidrolase          | 1 ± 0.7                       | 0.3 ± 0.2                         | 310 ± 260                       | 30.5                | 2.5 ± 0.9                    | 2.3 ± 0.8                               |
| N21-EcAII          | 13.5 ± 0.6                    | 3.3 ± 0.2                         | 87 ± 7                          | 8.5                 | 12.2 ± 1.3                   | 0.9 ± 0.1                               |
| N26-EcAII          | 12 ± 2                        | 2.9 ± 0.5                         | 95 ± 10                         | 9.1                 | 11.3 ± 0.8                   | 0.9 ± 0.1                               |
| C8-EcAII           | 9.7 ± 0.8                     | 2.5 ± 0.2                         | 160 ± 87                        | 9.9                 | 8.3 ± 0.7                    | 0.9 ± 0.03                              |
| C8-EcAII m         | 4.6 ± 0.8                     | 4.7 ± 0.8\(^b\) (1.2 ± 0.8)       | 0                               | 14.4                | 3.2 ± 0.5                    | 0.2 ± 0.03\(^c\) (0.7 ± 0.12)           |
| EcAII\(_{\text{cryst}}\)\(^d\) | 1.9                          |                                   |                                 |                     | 11.4                         |                                         |
| EcAII\(_{\text{cryst}}\)\(^e\) | 2.1                          |                                   |                                 |                     | 10.8                         |                                         |

“Specific activity of immobilized EcAII receptors (Ag) was monitored on 9 × 9 mm gold-coated glass slides. All other immobilization and detection measurements were performed in the P4SPR instrument using 20 × 12 mm gold-coated prisms. \(^a\)Detection using 150 μg mL\(^{-1}\) anti-EcAII antibody (Ab). \(^b\)Density or binding ratio of the monomer; values comparable to 4 monomers (1 equiv tetramer) are in parentheses. \(^c\)Unit cell dimensions for ECAII in the tetrameric form (PDB 3ECA). \(^d\)Unit cell dimensions for ECAII in the monomeric form (PDB 1INNS).

(Figure 5 and Table 2). In addition to being less sensitive (lower detection signal generated), Kidrolase led to poor sensing reproducibility (average RSD = 35%) compared with the His-tagged variants (average RSD = 5–20%) at all antibody concentrations tested. A positive correlation was drawn between the immunosensing signal (detection of antibody; Ab) and the surface coverage of EcAII receptors (immobilized antigen; Ag), with \( R^2 = 0.9571 \) at the high antibody concentration (150 μg mL\(^{-1}\)) and the oriented N-terminally tagged EcAII reliably providing the highest SPR signal (Figure 5A). At a lower antibody concentration (15 μg mL\(^{-1}\)), the correlation was not as strong (\( R^2 = 0.6102 \)).

To better understand the behavior of EcAII as an antigenic receptor for sensing, a second property was verified, namely, the sensing efficiency (efficiency of analyte recognition). This is defined as the number of analyte molecules (antibody; Ab) detected per molecule of the immobilized receptor (EcAII antigen; Ag) or the Ab/Ag binding ratio. Despite its low surface density and poor sensitivity, we observed that the native tetrameric EcAII (Kidrolase) afforded a significantly greater sensing efficiency than the tetrameric His-tagged EcAII. At 150 μg mL\(^{-1}\) antibody concentration, an average of 2.3 antibody molecules were detected per immobilized Kidrolase molecule (Ab/Ag binding ratio ≈ 2:1; Table 2). This ratio is 2 to 3-fold more efficient than for the tetrameric His-tagged EcAII variants, which bound only 0.9 antibody per molecule (Ab/Ag < 1:1). The lower Ab/Ag binding ratio of tetrameric His-tagged variants may be related to their higher density on the gold sensing surface than Kidrolase. Native EcAII has an average surface density of \( \sim 1.15 \times 10^{12} \) molecules cm\(^{-2}\) in the plane of the crystal lattice (PDB 3ECA), with an average distance between protein tetramers of \( \sim 11.4 \) nm, from center to center (c.t.c.). On the sensing surface, the density of the immobilized Kidrolase was \( \sim 7\)-fold lower (\( 1.6 \times 10^{11} \) molecules cm\(^{-2}\)) with the average c.t.c. distance between the immobilized tetramers \( \sim 2.7\)-fold greater \( (\sim 30.5 \) nm). On the contrary, the surface density of tetrameric His-tagged EcAII variants immobilized on the sensing surface was \( \sim 1.3 \) to \( 1.8\)-fold greater than in the crystal lattice, with c.t.c. distances between tetramers \( \sim 1.2 \) to \( 1.3\)-fold shorter. Their higher packing may be promoted by their 8- to 25-residue terminal linkers, allowing for some overlapping of the immobilized tetramers and may favor bivalent antibody binding between the tightly packed EcAII molecules (allowing each Fab domain to bind two distinct neighbor Ag molecules), consistent with the lower Ab/Ag binding ratio (<1:1) (Figure 5B and Table 2). This contrasts with the looser packing of Kidrolase molecules, which appears to provide additional space between Kidrolase tetramers for...
antibodies to bind, consistent with the observation of an Ab/Ag binding ratio as high as 2:1.

Despite a low surface density (c.t.c. distance 33% larger than in the crystal), monomeric C8-EcAII lost in sensing efficiency (Ab/Ag ratio ≈ 0.2) if one considers molar ratios because the tetrameric EcAII benefits from four Ab binding sites per Ag molecule. Nonetheless, the sensing efficiencies of monomeric and tetrameric C8-EcAII were similar if considering equal amounts of subunit molecules (Table 2).

At a lower antibody concentration (15 μg mL⁻¹), a similar trend was seen, yet the Ab/Ag ratio was always lower. Specifically, we observed an average of 1.3 antibody bound per Kidrolase tetramer (Ab/Ag binding ratio ≈ 1:1) and 0.2–0.3 antibody bound per His-tagged EcAII variant (Ab/Ag binding ratio ≈ 0.25:1) (Figure SB and Table 2).

Overall, the lower sensing efficiency of the tetrameric His-tagged EcAII receptors was more than compensated for by their greater surface density (coverage) and by their high reproducibility relative to Kidrolase. These factors ultimately afforded significantly greater sensitivity when detecting anti-EcAII antibodies in the serum (Figure SA).

■ CONCLUSIONS

We have examined the sensing properties of EcAII immobilized by various modes onto low-fouling SPR sensor chips for the detection of anti-EcAII antibodies in serum. We have determined that the native tetrameric structure, while being essential for activity, is not required for antibody recognition. Moreover, we showed that the extent of immobilization of EcAII was the main determinant of its immunosensing efficiency. Metal-coordination of His-tagged EcAII variants provided a significantly greater sensor coverage than covalent immobilization of native EcAII and therefore provided a greater sensitivity despite their reduced sensing efficiency per molecule. Moreover, metal chelation significantly improved the reproducibility of EcAII immobilization. This study illustrates the benefits of testing alternative immobilization strategies and highlights the positive impact of high receptor coverage and immobilization reproducibility toward obtaining a well-behaved sensing system.

■ EXPERIMENTAL SECTION

Materials and Reagents. The pharmaceutical drug Kidrolase (EUSA Pharma) was obtained as a lyophilized powder that contains 48.6% mass of glycine−NaOH, pH 6.8–7.0 and 51.4% mass of E. coli L-asparaginase II (EcAII) with an activity of 194.6 IU mg⁻¹. It was dissolved in a phosphate-buffered saline (PBS) buffer, pH 7.4 and prepared at a concentration of 0.1 mg mL⁻¹ (0.72 μM EcAII/0.65 mM glycine) for SPR analyses or was dialyzed against PBS to remove glycine. L-Glutamate dehydrogenase (NADP) from Proteus sp. was purchased from Sigma-Aldrich. L-Asparagine and δ-ketoglutaric acid were purchased from BioShop. NADPH tetrasodium salt was purchased from Calbiotech. The plasmid pET15b was purchased from Novagen. Human serum was purchased from Sigma. The E. coli l-asparaginase II (ansB) gene from E. coli K12 was obtained from the ASKA collection as a pCA24N-ansB construct.

His-Tagged EcAII Constructs. The full-length EcAII precursor ORF, including the signal peptide sequence (1044 bp), was amplified using polymerase chain reaction (PCR) from the pCA24N-ansB construct using the following primers (restriction sites are underlined): 5′-AAACATATGGAGTTTTTCAAAAAGACGGC-3′ (forward primer containing the NdeI restriction site) and 5′-AAAACTCGAGGTACT-GAGTTGAAGATCTGCT-3′ (reverse primer containing the XhoI restriction site) ligated into the similarly digested pET20b expression vector (Invitrogen). The resultant protein, named C8-EcAII, includes a C-terminal octapeptide His-tag (LEHHHHHH). To fuse an N-terminal His-tag to EcAII, the DNA sequence encoding the mature form of EcAII (978 bp) was amplified using PCR from the pET20b/ansB construct using the following primers: 5′-GAATTCCATATGGTACT-CAATATCACCTTTAGC-3′ (forward primer containing the NdeI restriction site) and 5′-CGGCTCGAGTACT-GATTGAAGATCTG-3′ (reverse primer containing the XhoI site). The ochre stop codon, TAA, was included (in bold in the reverse sequence). The ampiclon was digested with the corresponding restriction enzymes and ligated into similarly digested pET15b vector for N-terminal fusion with a sequence encoding 21 residues containing a His₆-tag, yielding the construct N21-EcAII. A second N-terminal fusion was constructed by introducing an enterokinase cleavage site between the mature EcAII sequence and the previous N-terminal fusion. The mature sequence was amplified from the pET15b/ansB construct with the forward primer: 5′-GAATTCCATATGGGACGACGACAGACAAGTACCATCCATTTTACG-3′ and the same reverse primer as for N21-EcAII. It was similarly ligated into pET15b, yielding the construct N26-EcAII. The DNA sequence encoding the enterokinase cleavage site is shown in bold in the forward sequence. Each ligation was transformed into competent E. coli BL21(DE3). The transformed cells were plated onto luira broth (LB)-agar containing ampicillin (Amp; 100 μg mL⁻¹), and the clones were selected and cultured in LB containing Amp (100 μg mL⁻¹) and stored at −80 °C in 25% GOH. The plasmids were isolated and the ORFs were sequenced at the IRIC Genomic Platform at Université de Montréal, using the T7 promoter and terminator primers. The DNA sequences were analyzed using the Clone Manager 9 (version 9.2) software.

Protein Expression and Purification. For N-terminally tagged EcAII (N21- and N26-EcAII), the cytosolic expression was performed as follows. Terrific broth (TB) (Amp 100 μg mL⁻¹) was inoculated with the appropriate GOH stocks and grown overnight at 37 °C and 230 rpm agitation. For the expression, 4 L flasks filled to 25% capacity with TB + Amp were inoculated with the appropriate precultures (1:1000 ratio), and the cells were grown at 37 ºC and 230 rpm, until the optical density at 600 nm reached 0.6. The protein expression was then induced overnight at 18 °C and 230 rpm by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced cells were harvested by centrifugation for 30 min at 3500 rpm using a SLA-3000 rotor. The cell pellets were stored at −80 ºC for 24 h, then thawed on ice, and resuspended in the lysis buffer (50 mM sodium phosphate, 10–20 mM imidazole, and 150 mM NaCl, pH 8) at a ratio of 10% (w/v). Lysozyme (final concentration of 1–2 mg mL⁻¹) was added, and the cells were placed on ice for 30 min, before clarification by sonication on ice for three 15 s cycles at 20 pulses/s. The cells were lysed using a Constant Systems cell disruptor (27 kPSI) cooled to 4 °C. The lysates were clarified from the cell debris by centrifugation for 30 min at 17.5k rpm using a SS-34 rotor and cooled to 4 °C. The supernatants were filtered through 0.2 μm filters, and the expressed His-tagged recombinant proteins were purified from the soluble fraction.
under native conditions by immobilized metal affinity chromatography (IMAC) (Ni-NTA) at 4 °C using a ÄKTA FPLC system equipped with a UPC-900 monitor and a P-20 pump system (GE Healthcare) and a 5 mL His-trap column (GE Healthcare). The nickel resin was equilibrated with 5 column volumes (CV) of the lysis buffer. The lysate was applied at a flow rate of 0.5 mL min⁻¹. After the recovery of the absorbance baseline at 280 nm, an imidazole gradient of 10–100 mM was applied over 3 CV and maintained at 100 mM for 3 CV before eluting with a jump to 500 mM imidazole. Fractions of 1 mL were collected. Following the analysis on 15% SDS-PAGE, the fractions containing EcAII were pooled, concentrated to 1 mL using an Amicon concentrator [molecular weight cutoff (MWCO) = 10 kDa] and applied at a flow rate of 0.5 mL min⁻¹ on a 90 mL Superdex 75 gel filtration column (1.6 × 55 cm) equilibrated with PBS, pH 7.4 at 4 °C. The collected 0.5 mL fractions corresponding to the major peak were analyzed using 15% SDS-PAGE, and the fractions of highest purity were pooled. The protein concentration was determined using the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) and Kidrolase as standards. The purified EcAII samples were diluted to 0.1–1 mg mL⁻¹ in PBS, pH 7.4, aliquoted, and flash-frozen over dry ice/ethanol for storage at −80 °C. Similar procedures were performed for purification of the C-terminally His-tagged C8-EcAII, with the following modifications. Periplasmic expression of C8-EcAII was performed in a ZYP-5052 autoinducing medium inoculated (1:100) with a preculture. Following growth at 37 °C for 2 h, the cultures were incubated at 20 °C for overnight expression. Following the application of the cell lysate onto the His-trap column, the elution was performed by a stepwise gradient from 20 to 250 mM. The purified protein was quantified, filter sterilized (0.2 μm), and flash-frozen for storage at −80 °C or kept at 4 °C.

Exact Mass Determination (LC–MS). The exact mass of the purified proteins (0.1 mg mL⁻¹ in PBS) was determined using electrospray ionization (ESI) mass spectrometry on a LC–MS time of flight (TOF) (Agilent) spectrometer at the Regional Mass Spectrometry Centre at Université de Montréal.

L-Asparaginase Activity Measurements. The hydrolysis of L-Asn catalyzed by EcAII was assessed using spectrophotometry at 37 °C by monitoring ammonia production as an end-point assay using direct Nesslerization or using a continuous coupled assay with GDH. Each reagent was freshly prepared in the reaction buffer (modified PBS: sodium phosphate concentration increased to 50 mM and pH adjusted to 8 before the experiment).

Direct Nesslerization. A standard curve for ammonia concentration was generated with the (NH₄)₂SO₄ concentration ranging from 0 to 5 mM in PBS, pH 8 or Tris-HCl, pH 8.6. The EcAII reactions (1 mL) were performed in the same buffer with 5 μg of l-asparaginase and 9 mM l-Asn. Each reaction was performed for 30 min at 37 °C and then quenched with 0.05 mL of 1 M trichloroacetic acid. A total of 0.1 mL of the reaction solution was used for revelation with 0.25 mL of Nessler reagent and 2.15 mL of water (final volume of 2.5 mL), and the absorbance was measured at 450 nm. The enzyme specific activity was determined based on freshly generated standard curves.

Coupled assay. The ammonia produced upon l-Asn hydrolysis by EcAII served as the substrate for the second enzyme GDH in a coupled reaction that converts α-ketoglutarate into glutamate with the oxidation of NADPH into NADP⁺. The reaction rate was observed in a continuous manner by monitoring the decrease in the NADPH absorbance at 340 nm, using εNADPH = 6.22 mM⁻¹ cm⁻¹. The parameters of the coupled assay were optimized regarding the concentration of GDH and each substrate. To this effect, the affinity and the catalytic efficiency of GDH for ammonia were determined under saturated concentrations of NADPH (250 μM = 10 × Kₘ) and α-ketoglutarate (175 mM = 17 × Kₘ) and variable concentrations of NH₄Cl (0–50 mM) (Table S1). The ammonia produced by EcAII at a saturated concentration of freshly prepared L-Asn (5 mM ≈ 500 × Kₘ) was monitored using the coupled GDH assay under the saturated conditions described above (freshly prepared substrates) with various amounts of EcAII (0.1–10 μg). The maximal rate of NADPH oxidation (maximal GDH velocity) was then plotted as a function of EcAII loading. The slope of the linear portion of the curve (dynamic range) observed from 0.1 to 1 μg EcAII was taken as the apparent specific EcAII activity (μmole NADPH oxidized/min per mg of EcAII) under the assay conditions. We refer to this value in terms of units (U mg⁻¹ EcAII) in the coupled assay.

SEC. The oligomerization states of native EcAII (Kidrolase) and each His-tagged EcAII were analyzed using analytical SEC using an ÄKTA FPLC system. Different protein concentrations (0.4 mL injections) were applied onto a calibrated 24 mL size exclusion column (GE Superdex 200, 10/300 mm) equilibrated with PBS, pH 7.4 at a flow rate of 0.5 mL/min in PBS, pH 7.4 at 4 °C. The EcAII oligomeric forms were determined by correlating the elution volume (Vₑ) of each peak with both the expected MW according to the elution volume of protein standards and the Stoke’s radius. The calculated accessible surface area (ASA) reported for the monomer is 14 000 Å² but only 38 500 Å² for the tetramer. When considering the Stoke’s radius of the calibration standards, the calculated Stoke’s radius of the EcAII tetramer (39 Å) is consistent with the measured Stoke’s radius both in crystal form (32 Å) and in solution (30.3 Å).

Native-PAGE. Protein preparations were concentrated to 2.5 mg mL⁻¹ in PBS, pH 7.4 using Amicon concentrators (MWCO = 10 kDa) and analyzed using CN-PAGE and BN-PAGE using Novex NativePAGE 4–16% Bis-Tris gels (1.0 mm), pH 7. For BN-PAGE, the protein samples were prepared in a loading dye consisting of 50 mM Bis-Tris, 500 mM ACA, 10% G2H, and 5% Coomassie blue G-250 and applied to 4–16% Bis-Tris gels or 10% Bis-Tris gels. Native electrophoresis was performed with deep or light blue cathode buffer [50 mM tricine, 15 mM Bis-Tris with 0.02% (deep) or 0.001% (light) Coomassie blue G-250] and anode buffer (50 mM Bis-Tris), both adjusted to pH 7. Electrophoresis under light blue conditions was referred to as LBN-PAGE. For CN-PAGE, the Coomassie blue G-250 loading dye was replaced by bromphenol blue with or without ACA. In addition, a clear cathode buffer was used (without G-250). Electrophoresis was performed for 2 h at 90–100 V. CN-PAGE gels were stained with Coomassie brilliant blue R-250. CN-PAGE and BN-PAGE gels were destained with 10% acetic acid and 45% methanol.

CD. The CD spectra of Kidrolase and each His-tagged EcAII were recorded using a Chirascan spectropolarimeter (Applied Photophysics). Far-UV CD spectra (190–250 nm) were recorded under a nitrogen atmosphere at 25 °C in PBS, pH 7.4 with a protein concentration of 0.1 mg mL⁻¹ in a 1 mm quartz cuvette. The scans were performed with a step of 0.4 nm (3.6 s/point) and a bandwidth of 1 nm. The spectra were
corrected from the background (buffer). The data were converted into molar ellipticity ($\theta$). The unfolding curve is given by the following equation.51 The chemical denaturation of EcAII may proceed via the intimate dimer intermediate ($N \rightarrow 2I \rightarrow 4U$),50 the thermal denaturation was treated as an apparent two-state process, consistent with the overall shape of the unfolding curves. We thus determined the apparent melting temperature by fitting to a two-state model ($N \rightarrow D$), where denaturation operates between the fraction of native ($f_N$) and denatured ($f_D$) molecules and where $f_N + f_D = 1$. In this model, the fluorescence signal value ($y$) at any point of the unfolding curve is given by the following equation.51

$$y = y_N f_N + y_D f_D$$

(1)

The values $y_N$ and $y_D$ correspond to the fluorescence intensity for the native and denatured states, respectively. By combining these equations, the fraction of denatured molecules at any value of $y$ (fluorescence intensity) is obtained by the following equation

$$f_D = (y_N - y)/(y_N - y_D)$$

(2)

The denaturation equilibrium constant can be calculated as follows

$$K = f_D/(1 - f_D) = f_D/f_N = (y_N - y)/(y - y_D)$$

(3)

The unfolding free energy change can be calculated as follows

$$\Delta G = -RT \ln K$$

(4)

The melting temperature ($T_m$) can be obtained by plotting the unfolding free energy ($\Delta G$) as a function of temperature, where $T_m$ corresponds to the temperature where $f_N = f_D$ and the unfolding free energy is null ($\Delta G = 0$). We noted that, beyond the inflection point, some aggregation was observed.

**Fluorescence Spectroscopy.** Intrinsic fluorescence measurements were performed using a Varian Cary Eclipse spectrophotometer. Fluorescence spectra were recorded from 287 to 450 nm after excitation at 278.5 nm (at a protein concentration of 0.05 mg mL$^{-1}$ in PBS, pH 7.4 in a 1 cm quartz cuvette. Thermal denaturation was performed from 20 to 90 °C (0.5 °C/min) using a Peltier temperature controller. Melting curves were generated by plotting the fluorescence intensity at any point of the unfolding curve, some aggregation was observed.

The melting temperature ($\Delta G$) was fitted to a two-state model ($N \rightarrow D$), where denaturation operates between the fraction of native ($f_N$) and denatured ($f_D$) molecules and where $f_N + f_D = 1$. In this model, the fluorescence signal value ($y$) at any point of the unfolding curve is given by the following equation.51

$$y = y_N f_N + y_D f_D$$

(1)

The values $y_N$ and $y_D$ correspond to the fluorescence intensity for the native and denatured states, respectively. By combining these equations, the fraction of denatured molecules at any value of $y$ (fluorescence intensity) is obtained by the following equation

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The denaturation equilibrium constant can be calculated as follows

$$K = f_D/(1 - f_D) = f_D/f_N = (y_N - y)/(y - y_D)$$

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The melting temperature ($T_m$) can be obtained by plotting the unfolding free energy ($\Delta G$) as a function of temperature, where $T_m$ corresponds to the temperature where $f_N = f_D$ and the unfolding free energy is null ($\Delta G = 0$). We noted that, beyond the inflection point, some aggregation was observed.

**ELISA.** MaxiSorp microplates (Nunc-Immuno plate, Thermo Scientific, cat no. 62409-50) were coated with 0.1 mL of native or His-tagged EcAII (10 µg mL$^{-1}$) diluted in 0.05 M carbonate/bicarbonate, pH 9.5 and incubated overnight at 4 °C. ELISA assays were performed, as reported by Wang and co-authors,12 with the following modifications: the primary rabbit polyclonal anti-EcAII antibody (IgG) ANSZ (Antibodies Online, cat no. ABIN95396) was resuspended in human serum (Sigma, cat no. H4522) and diluted in PBS, pH 7.4 to concentrations ranging from 0.1 pM to 1 µM for calibration, assuming a MW of 150 kDa. Polyclonal HRP-conjugated goat antirabbit IgG (Abcam, cat no. ab97200) at a dilution of (1:1000) in PBS was used for secondary detection. A volume of 0.1 mL of freshly made 0.4 mg mL$^{-1}$ o-phenylenediamine dihydrochloride (OPD) prepared in 0.1 M citrate buffer, pH 6 containing 0.02, 0.16, or 3% hydrogen peroxide (high, intermediate, and low sensitivity conditions, respectively) was added to the wells and incubated for 30 min in the dark, after which the reaction was stopped by the addition of 0.1 mL of 1 M phosphoric acid. The anti-EcAII antibody concentration was measured by monitoring the absorbance at 490 nm (specific product absorption). The absorbance at 490 nm was then subtracted from the absorbance at 650 nm to control for nonspecific adsorption. Oriented His-tagged EcAII were further analyzed using ELISA using Pierce Nickel-coated plates. Calibration was performed with the ANSZ antibody from Antibodies Online and was confirmed with a rabbit polyclonal anti-asparaginase II antibody (IgG) from Novus Biologicals (NB100-66516; not shown). The titration curves were fitted to a logarithmic function, and the dissociation constant was measured using multiple binding sites analysis using the GraphPad Prism 6.0 software.

**Sensor Chip Fabrication.** Sensor chips were constructed by depositing a thin gold film on either 9 × 9 × 0.5 mm glass slides for monitoring the extent of immobilization and on-chip asparaginase activity or on 20 × 12 × 3 mm glass prisms for immunosensing in a portable P4SPR instrument (Affinité Instruments) that has been described in a previous report.19 Sensing surfaces were prepared by depositing chromium (~0.7 nm thick) and then gold (~50 nm thick) on the glass surface using a Cressington 308R sputter coater (Tel Pella Inc.). SPR sensing experiments (wavelength interrogation) were performed in Kretschmann configuration. The gold surfaces were immersed in a 1 mg mL$^{-1}$ solution of 3-MPA-LHDLHD-OH peptide in dimethylformamide (DMF) to form a SAM that prevented surface fouling.22,23 The terminal carboxylates on the SAM remain free, to covalently immobilize native EcAII by cross-linking with its surface-exposed lysine residues. For immobilization of EcAII by coordination of terminal His-tags, the SAM was functionalized with NTA-Co to yield Au-MPA-LHDLHD-NTA-Co, as previously described.17

**On-Chip Measurement of Activity and Extent of Immobilization.** The specific activity of immobilized EcAII was monitored on the 9 × 9 mm gold-covered chips. The SAM-functionalized chips were placed on a dove prism (above a thin layer of immersion oil) and sealed with a rubber ring fitted in the injection module. The chip surface was rinsed with water and then with the buffer (PBS, pH 7.4). Following the adjustment of the plasmonic band (typical minimum at ≈ 620 nm), the baseline was set in S polarization, and the reference was collected (average of 10 scans per spectrum for a total of 100 spectra). The sample wavelength shifts ($\Delta \lambda_{SPR}$) were recorded in P polarization. The native EcAII was surface-immobilized by cross-linking its surface-exposed lysines to the free carboxylates of the SAM, using a previously-reported EDC/NHS cross-linking procedure.53 The chip was rinsed with at least 6 mL of the buffer for 2 min, and 1 mL of EDC/NHS (1:1 mixture with a final concentration of 200 and 100 mM, respectively) was injected onto the peptide surface. PBS, pH 4.5 was injected to activate the surface (for 2 min), and 0.8 mL of 0.1 mg mL$^{-1}$ native EcAII (Kidrolase) was injected. Surface immobilization was monitored for 20 min before the surface was washed with 6 mL of PBS. Immobilizing the His-tagged EcAII onto NTA-Co-functionalized SAM was performed as above, with the exception of the EDC/NHS and the PBS, pH 4.5 injection steps.

The surface coverage ($\Gamma$, ng cm$^{-2}$) of the immobilized native or His-tagged EcAII was calculated from the change in the wavelength ($\Delta \lambda_{SPR}$) upon immobilization of EcAII using the following equation

$$\Gamma = \rho(-I_l/2) \ln(1 - (\Delta \lambda/m(n_{SAM} - n_{medium})))$$

(5)
where \( \rho \) corresponds to the density of the adsorbed protein monolayer (1.3 g cm\(^{-2} \)), \( l_p \) is the plasmon penetration distance (~230 nm), \( \Delta \) is the shift in the wavelength associated with protein immobilization, \( m \) is the refractive index sensitivity of the SPR sensor (1765 nm/RIU), \( n_{\text{SAM}} \) is the refractive index of the peptide SAM (1.57 RIU), and \( n_{\text{medium}} \) is the refractive index of the buffer (1.33476 RIU). The total amount of immobilized EcAII on the sensing surface \( (Q) \) was determined using the formula \( Q = \Gamma S \), where \( S = 0.166 \) cm\(^2\) in contact with the protein.

To monitor the activity of the surface-immobilized EcAII, the 9 x 9 mm EcAII-coated chips were placed upright along the side wall of a UV/vis quartz cuvette. The chips were immersed in a solution containing 5 mM L-Asn, 17 K\( \alpha \)-ketoglutarate, 10 K\( \alpha \) NADPH, and 1.0 IU GDH in a modified PBS buffer, pH 8 (as described for asparaginase activity measurements) with slow agitation. The activity of the immobilized EcAII was measured by monitoring the change in the absorbance at 340 nm over 240 min because of the oxidation of NADPH accompanying the consumption of ammonia by GDH. The maximal GDH velocity was corrected from the blank (no EcAII) and was used to determine the activity of the immobilized EcAII. The specific activity (U mg\(^{-1}\)) of the immobilized EcAII was determined according to the mass of the immobilized protein \( (Q) \) on the chip.

**SPR Immunosensing.** SPR immunosensing experiments were performed using the P4SPR portable instrument (Affiniti Instruments). The 20 x 12 mm dove prisms coated with gold and functionalized with the appropriate antiifouling SAM (as described above, either with or without NTA-Co) were placed in the P4SPR instrument for EcAII immobilization. The baseline from 1 mL of PBS was recorded for 2 min, and native or His-tagged EcAII (0.4 mL of 0.1 mg mL\(^{-1}\) protein) was injected as above, followed by rinsing with 1 mL of PBS for 2 min. Following immobilization, 0.4 mL of blank human serum was injected over 10 min to passivate the surface. Human serum spiked with different concentrations of polyclonal rabbit anti-asparaginase antibodies (0.4 mL) was then injected, and antibody binding was monitored for 20 min. Calibration of the sensor was performed with serial injections of increasing concentrations of anti-asparaginase antibodies on a single sensor chip, as previously described. The SPR shifts were calculated with MATLAB software and served to calculate the surface density of the analyte bound onto the immobilized antigenic receptors, as described above. Taking into account the exact MW of each EcAII variant, we calculated the number of EcAII molecules immobilized on the sensing surface (molecule cm\(^{-2}\)). Calculation of the number of antibody molecules bound to antigen was based on a MW of 150 kDa for the antibody. Statistical analysis of the variance was performed according to Tukey’s multiple means comparison test identified by one-way ANOVA. Dimensions of the unit cell of tetrameric EcAII in crystal form (PDB 3ECA: \( \alpha = 7.6 \times b = 9.6 \times c = 11.1 \) nm; \( \beta = 90^\circ \), \( \gamma = 90^\circ \)) allowed estimating an average footprint of 80 nm\(^2\) (8 x 10\(^{-15}\) cm\(^2\)) per monomer with an average c.t.c of 10.8 nm in the plane of the crystal lattice.
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