The impact of antibody/epitope affinity strength on the sensitivity of electrochemical immunosensors for detecting small molecules

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Abstract A displacement immunoassay involves having a labelled analogue of the analyte (the epitope) already bound to the antibody. The presence of the analyte causes a competition for antibodies, and some of the antibodies dissociates from the epitope so that it can bind with the analyte. Herein, the influence of the affinity of the surface-bound epitope for the antibody on the sensitivity and selectivity of a displacement immunosensor is explored both theoretically and experimentally. An electrochemical immunosensor described previously [1], where the dissociation of antibodies from an electrode surface causes an increase in current from surface-bound ferrocene species, is used for this purpose. As expected, the ease and effectiveness of the bound antibody being displaced is inversely related to the affinity of the antibody to the surface-bound epitope relative to the analyte in solution as expected. However, if the affinity constant is too low, selectivity and/or sensitivity are compromised. Experimental results are qualitatively compared with a simple mass-action model.

Keywords Immunosensor · Displacement assay · Theory · Electrochemistry

Introduction

A displacement immunosensor is one where the antibody and antigen are already complexed to each other on a surface prior to exposure to the sample [2–8]. Exposure to the sample causes some dissociation of this complex as antibodies bind to the target analyte with a concomitant analytical signal produced. The great advantage of the displacement assay is that all the required reagents for the immunosensors can be associated with the surface; hence, the immunosensor can be user-intervention-free with no addition or rinsing steps required. Nevertheless, displacement assays where antibodies are dissociated from a surface are seldom employed in immunosensors because the assay faces the challenge of actually displacing the bound antibody efficiently from the surface-bound epitope. The issue arises because for immunoassays to be effective requires a high affinity between the antibody and the species to which it binds. However, this high affinity makes dissociation of the antibody from its binding partner unfavorable. Furthermore, with many surfaces employed in sensing, the rest of the surface, apart from the epitope, also has some affinity for the antibody, making it even more difficult to remove.

In response to this challenge, we have developed a user-intervention-free electrochemical immunosensor for small molecules based on an immunoassay [1, 9, 10] depicted in Fig. 1. With this immunosensor, a mixed layer of molecular wire (MW), to which a redox reporter (a ferrocene derivative) and the epitope to, which an antibody complexes, are attached, and oligo(ethylene glycol) (OEG) antifouling layer

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Fig. 1 Schematic of an electrochemical immuno-biosensor: **a** shows the electrode surface after the antibody/epitope complexation with the corresponding attenuated electrochemical signal; **b** shows the detection

of small analyte via a displacement assay where the antibody is released with the expected increase in electrochemistry against the incubation time

(Fig. 1a). Transduction in this system is based on the amperometric signal of surface-bound ferrocene moiety being attenuated when antibodies bind to the epitope. The attenuation of the surface electrochemistry is attributed to the immersion of the ferrocene into a protein environment. When this immunosensor is exposed to a sample containing a small molecule analyte, such as a drug [10], displacement of antibody can proceed. The presence of the analyte in solution results in a competition for surface-bound antibodies between the surface-bound epitope and the analyte. The result is some antibody dissociates from the sensing surface and, in this case, causes an increase in electrochemical current. The dissociation of antibodies from the electrode is assisted by the surface being comprised of a protein-resistant layer, apart from the epitopes to which the antibody binds [1], and by using repulsive electric fields to promote dissociation of the antibody from the epitope [9, 11, 12].

The question this paper seeks to address is what impact does the antibody/epitope affinity have on the performance of an immunosensors based on a displacement assay? The impact of the affinity constant between the surface-bound epitope and the antibody on the

sensitivity and selectivity of the final immunosensor is an issue that relates to all displacement assays that has yet to be explored in detail. There has been one theoretical study of displacement assays by Yasuura et al. [13] where a transient model of a displacement assay was described for an immunosensor for trinitrotoluene that employs surface plasmon resonance for transduction [14]. As this device was in a flow cell, the model needed to consider mass transport, and so, the two layers were defined with partitioning between these two layers, one where solution flowed through the flow cell and the other was a static boundary layer where mass transport was by diffusion only. The necessity for mass transport is exemplified by the fact that, even in the absence of analyte, some antibody displacement was observed. What this theory showed was that the equilibrium constant for the dissociation of antibody from the surface was the major factor determining the sensitivity of the immunosensor (herein, we use the term sensitivity according to the IUPAC definition of sensitivity being the ability to determine between small changes in concentration as distinct from detection limit as is so commonly used in the immunoassay literature) while the equilibrium constant between the antibody and

the analyte was shown to have a large influence on the sensors's dynamic range (and hence detection limit).

Therefore, the purpose of this paper is to use our electrochemical immunosensor format to explore both theoretically and experimentally the influence of the surface-bound epitope on the performance of displacement assay-based immunosensors. This was achieved using six different species of structurally related fluoroquinolones, namely ciprofloxacin, norfloxacin, enofloxacin, danofloxacin, ofloxacin, and enrofloxacin as surface-bound epitopes for the detection of enrofloxacin (Fig. S1). Epitopes with similar molecular structures have different affinity constants for a given specific antibody [15–17]. The influence of these different epitopes was accessed from the perspectives of sensitivity, limit of detection, and dynamic range.

Theoretical framework

As stated in the “Introduction” the purpose of this paper is to understand what variables influence the analytical performance of a displacement immunosensor. The analytical signal in immunosensors as depicted in Fig. 1 relate to the amount of antibody that is displaced from the immunosensing surface. The amount of antibody that is displaced, however, is partly determined by how much antibody is complexed with the surface in the first place. Hence, to fully understand a displacement, immunosensor requires an understanding of both the loading of the surface with antibodies and the displacement of these antibodies. The equilibrium equation describing a displacement of surface-bound antibodies when analyte is being detected is represented by

where $[E_x]$ is the concentration of the surface epitope, $[Ab]$ the antigen in solution, and $[E_x:Ab]$ and $[Ag:Ab]$ are the epitope/antibody and antigen–antibody complexes, respectively. The complexation of Ab with E_x to give a surface complex of concentration $[E_x:Ab]$ is also an equilibrium reaction where

The amount of antibody that is dissociated from the surface is given by

From the two underlying equilibria, it is apparent that a high K_1 is imperative for a high occupation of surface-bound antibody binding sites (the epitope) because the analytical signal is derived from surface-bound antibody being

displaced, that is, a high occupation of binding sites is important for high sensitivity. However, the higher the affinity, the harder it will be to displace the epitope from the antibody binding sites and, potentially, the lower the sensitivity of the final device. This is because, for effective displacement of epitope, K_3 needs to be higher than K_1 . Hence, a balance is required between the affinity for the epitope and the affinity for the antigen (analyte).

Antibody–antigen interactions can, in many ways, be thought of as analogous to host–guest chemistry [18, 19]. Particularly relevant to this work here is the situation described for classical competition equilibrium such as those used in indicator-displacement assays popular in optical measurements [19, 20]. In an indicator-displacement assay, a labelled guest is displaced from a host via the presence of an unlabelled guest. The release of the labelled guest provides the signal. This is the equivalent situation to a displacement-based immunosensor, and hence, the simple mass-action models to describe an indicator-displacement assay can be used for displacement immunosensors.

The expressions for the latter two equilibriums are shown in Eqs. 4 and 5.

$$K_1 = \frac{[E_x : Ab]}{[E_x][Ab]} \quad (4)$$

$$K_3 = \frac{[Ag : Ab]}{[Ag][Ab]} \quad (5)$$

The laws of mass action can be used to describe the quantification of a displacement assay. A number of assumptions need to be made as shown below.

1. Mass transport is not significant. This is justified as in static solutions, with the high affinity of the antibody for the surface-bound epitope, the antigen in solution will be in excess of the amount of antibody near the sensing interface, and hence, mass transport can be ignored in the model.
2. Equilibrium constants are not influenced by the solid surface. In other words, $K_3 = K_1$ when the epitope and antigen are the same species.
3. The antibody is homogeneous and has a single binding site that recognizes only one epitope on the antigen.
4. The epitope is homogeneous.
5. The epitope/antibody and antibody–antigen reactions are at equilibrium.
6. The separation of free from bound antibodies is complete.
7. There is no nonspecific binding between antibody/epitope.

8. Before a displacement assay, $[Ex]_0 = [Ab]_0 = [Ex:Ab]_0$.

To show the impact of K_1 on the performance of a displacement assay involves solving the cubic Eq. 13 the derivation of which is given in Eqs. 6–12 in the [Supplementary Information](#).

$$[Ab]^3(K_1K_3) + [Ab]^2\{K_1 + K_3 + K_1K_3([Ag]_0 + [Ex]_0 - [Ab]_0)\} + [Ab](1 + K_1[Ex]_0 + K_3[Ag]_0 - K_1[Ab]_0 - K_3[Ab]_0 - [Ab]_0) = 0 \quad (13)$$

Equation 13 contains five known variables ($[Ab]_0$, $[Ag]_0$, $[Ex]_0$, K_1 , and K_3). This cubic equation has three solutions that may or may not include complex numbers. The smallest positive real solution is the only one of relevance here, and it can be for instance obtained by the root solver algorithm in Matlab®. Once the concentration of $[Ab]$ is known, the concentration of $[Ex]$ can be calculated from Eq. 9 in the [Supplementary information](#) which is then used to calculate the mole fraction of unbound epitope according to Eq. 14.

$$f_{Ex} = \frac{[Ex]}{[Ex]_0} = \frac{1}{1 + K_1[Ab]} \quad (14)$$

Experimental methods

Reagents and materials

The aniline derivatives, from which the aryl diazonium cations, OEG and MW, were formed, were custom-synthesized following reported procedures [21, 22]. The synthesis of the MW had been modified such that there was a carboxylic acid moiety at the distal end of the molecule and has been described in detail previously [23]. The 1,1'-di(aminomethyl)ferrocene (FDMA) was synthesized according to Ossola et al. [24]; 1,3-dicyclohexylcarbodiimide (DCC), absolute ethanol, $NaNO_2$, ciprofloxacin, norfloxacin, ofloxacin, enrofloxacin, danofloxacin and enrofloxacin were obtained from Sigma (Sydney, Australia). The commercially available monoclonal anti-enrofloxacin IgM antibody was purchased from Auspep Pty Ltd. (Sydney, Australia). All buffer solutions were prepared with Milli-Q™ water with resistivity of 18.2 MΩcm (Millipore, Sydney, Australia). Phosphate-buffered saline (PBS) was made up by 0.137 mol L⁻¹ NaCl and 0.1 mol L⁻¹ $NaH_2PO_4 \cdot 2H_2O$, and adjusted to pH 7.4. Phosphate buffer was made up by 0.05 mol L⁻¹ KCl and 0.05 M K_2HPO_4/KH_2PO_4 and adjusted to pH 7.0.

Electrochemical measurements

All electrochemical measurements were performed with a BAS-100B/W electrochemical analyzer (Bioanalytical Systems Inc., USA). Electrodes were 3-mm-diameter glassy carbon disk electrodes (Bioanalytical Systems Inc., USA) used as the working electrodes, a platinum flag (5 mm²) as the counter, and a Ag/AgCl (NaCl, 3 M) reference electrode, relative to which all potentials were quoted. Glassy carbon electrodes were polished successively in 1.0, 0.3, and 0.05 μm alumina slurries (5 min each) of alumina in Milli-Q water on microcloth pads (Buehler Lake Bluff, IL, USA). Before derivatization, the glassy carbon electrodes were dried with an argon stream. The association/dissociation of the antibody to/from the surface-bound epitope was monitored by square wave voltammetry (SWV), measuring the current versus potential profile (+0.8 to 0.1 V) in the negative direction. For the SWV measurements, the parameters used were step potential=4 mV; frequency=15 Hz; and amplitude=25 mV.

Construction of an electrochemical immuno-biosensing interface

The procedures used for glassy carbon electrode surface modification with mixed layer of in situ-generated MW/OEG 1:50 aryl diazonium cations and further modification with FDMA were similar to the works recently published [9, 10]. The 1,1'-di(aminomethyl)ferrocene modified glassy carbon electrodes were then incubated in an absolute ethanol solution containing 2 mmol L⁻¹ of one of enrofloxacin, ciprofloxacin, norfloxacin, enrofloxacin, danofloxacin, or ofloxacin and 40 mmol L⁻¹ DCC for overnight at room temperature to attach epitope to the free amine on the surface-bound FDMA. The epitope-modified electrodes were rinsed with copious amounts of distilled ethanol followed by Milli-Q™ water, scanned by CV until stable current versus potential achieved, and finally dried with argon. At this point, construction of the affinity surfaces for the specific recognition of monoclonal anti-enrofloxacin IgM antibody was accomplished.

For measuring the different antibody/epitope affinities (this value is regarded as IC₅₀) from the antibody titration plot, serial concentrations of anti-enrofloxacin antibody (ranging from 0.4 ng mL⁻¹ to 40 μg mL⁻¹ in PBS, pH 7.4) were prepared. Fluoroquinolone-modified electrodes were incubated for 30 min at room temperature in 50-μL aliquots of monoclonal anti-enrofloxacin IgM antibody. After the antibody binding, the electrode surface was rinsed with copious amounts of Milli-Q™ water to remove weakly bound antibody. A serial concentration of analyte enrofloxacin (ranging from 0.1 ng mL⁻¹ (0.3 nmol L⁻¹) to 31.6 μg mL⁻¹ (87.9 μmol L⁻¹) in PBS, pH 7.4) was prepared to obtain the displacement assay calibration plots. The anti-

enrofloxacin IgM antibody modified electrode was exposed to the desired concentration of free enrofloxacin solution. SWV measurements were recorded at 0, 2, 4, 6, 8, and 10 min, respectively, to monitor the dissociation of the antibody from the surface-bound epitope. Note that every single measurement required a new electrode to be prepared from bare glassy carbon, as each electrode could be used only once.

Results and discussion

Theoretical results

As outlined in the “Theoretical framework” section, there are two main equilibria in question that are expected to influence the analytical performance of the final immunosensors, K_1 and K_3 . K_1 is important in both the loading of the surface with antibody and the displacement of antibodies from the sensing surface while K_3 is important with regards to the displacement of antibodies alone. We will discuss the loading of the immunosensing interface and then displacement in turn.

Loading of the immunosensing interface

The impact of K_1 on the amount of antibody that binds to the immunosensing interface as a function of $[Ab]$ in solution is shown in Fig. 2. The y-axis here is represented as the amount of free epitope $[E_x]$ as this represents the amount of free binding sites for antibodies. Also shown on the right y-axis is the current, I , in arbitrary units to represent the

Fig. 2 The changes in the level of unbound epitope and current (I) when the concentration of antibody is increased from $[Ab]=0$ to $[Ag]=10^{-6}\text{molL}^{-1}$ versus different K_1 (in liters per mole) as indicated by the legends on the binding isotherms. In all cases, $[E_x]_0$ is fixed at 10^{-9}molL^{-1} , and there is no antigen present ($[Ag]=0$)

actual experimental electrochemical assay where, as antibodies bind to the surface, the current of the surface-bound ferrocene species is attenuated. Hence, a decrease in the fraction of $[E_x]$ decreases I . For simplicity, in this and all the subsequent simulations below, the initial concentration ($[E_x]_0$) of the surface epitope is assumed to be 10^{-9}molL^{-1} .

Figure 2 shows the expected increase in the fraction of filled binding sites as the concentration of antibody in solution during the loading step is increased, and hence, the greater the decrease in current. More important is the impact of K_1 . As can be seen, with low values of K_1 (below 10^8L mol^{-1}), even at 10^{-6}mol L^{-1} antibody, the surface is not completely saturated with antibody. In contrast, very high K_1 (above 10^{11}L mol^{-1}) has very little impact of K_1 on $[E_x:Ab]$ formed. The general trend here is however that the higher K_1 , the higher the amount of $[E_x:Ab]$ for a given concentration of antibody. The importance of this trend is that the amount of antibody bound to the immunosensing interface defines the analytical sensitivity. Hence, K_1 is the determining factor in the sensitivity of the immunosensor.

Displacement assay

The displacement of antibodies from the immunosensing interface for a given concentration of analyte will be dictated but the affinity of the antibodies for both the antigen in solution (the analyte) and the surface-bound epitope. That is, both K_1 and K_3 will be important in determining the extent of antibody dissociation from the surface. The relationship between K_1 , K_3 , and f_{Ex} is shown in Fig. 3a when the antigen concentration is fixed equal to the $[E_x]_0$ and $[Ab]_0$ at 10^{-9}molL^{-1} . The change (first derivative) in the fraction of unbound epitope f_{Ex} as a function of antigen concentration when K_1 and K_3 are equal are also shown in Fig. 3b.

The key message of Fig. 3a is that it is the ratio of K_3/K_1 that is important in determining whether antibody will be displaced from the surface of the immunosensing interface when antigen is in solution. As can be seen from Fig. 3a, when K_1 is high compared with K_3 , little or no antibody is displaced. Whereas when K_3 is high compared with K_1 , virtually all antibodies are displaced from the surface. It is only when K_1 and K_3 are within a couple of orders of magnitude from each other do small changes in either equilibrium constant have a significant impact on the analytical performance of the immunosensor at this concentration. The simulation data also in Fig. 3b shows that the sensitivity (largest gradient) of the immunosensor is highest when the antigen concentration is lower than the surface epitope concentration (here 10^{-9}molL^{-1}), provided of course that K_1 and K_3 are at reasonably large ($>10^8\text{Lmol}^{-1}$).

Of interest to the experimentalist is how the ratio of K_3/K_1 influences the calibration response of the immunosensor. That

Fig. 3 The level of unbound epitope with $[E_x]_0$ and $[Ab]_0$ is fixed at $10^{-9} \text{ mol L}^{-1}$; **a** as function of $\log K_1$ and $\log K_3$ with the concentration of antigen fixed at $[Ag]=10^{-9} \text{ mol L}^{-1}$. The labels *a-b*, *c-d* and *e-f* refer to the corresponding plots in Fig. 4. **b** The first derivate of the

changes in the level of unbound epitope with respect to the change (gradient) in the level of antigen ($d[Antigen]/[Antigen]$) with equal $\log K_1$ and $\log K_3$ plotted against $\log[Antigen]$

is, how does K_3/K_1 influence the performance of the immunosensor as a function of antigen concentration? This is shown in Fig. 4a–f for three different ratios of K_3/K_1 , as marked on the response surface in Fig. 5a. As K_3 is the affinity constant between antibodies and the antigen, it represents the affinity constant for the detection of the analyte and may not in fact be a variable that the experimentalists can alter. However, K_1 being the affinity constant of the antibodies to the immunosensing interface is a variable that can easily be altered simply by changing the surface-bound epitope. How the value of K_1 influences the calibration curves for a given K_3 of $10^{-8} \text{ mol L}^{-1}$ is shown Fig. 3b. As K_1 increases, the calibration curve is displaced to the right as higher concentrations of antigen in solution are required to displace antibodies. This is perhaps not a surprising result. Perhaps, more surprising, however, is that, as K_1 increases, the magnitude of the change in I is greater. This is because, as was illustrated in Fig. 2, at higher values of K_1 , more antibody complexes with the immunosensing interface than lower values of K_1 . This is most apparent at a K_1 value of 10^{-8} where the fraction of free epitopes, f_{Ex} , is above 0.9 at $[Ag]=10^{-12}$. An increase in K_3 , however, shifts all the calibration curves for different values of K_1 to lower concentrations. The message here is that the higher the value of K_3 for a given value of K_1 , the lower is the dynamic range. A further message is to choose as high a value of K_1 as possible, for higher sensitivity, but choose a value of K_3 that is significantly higher than K_1 , if possible. Note that these findings are consistent with the previous theoretical study by Yasuura et al. [13].

The effect of going from a low to high value of K_1 is shown in progression on the left side of Fig. 4, going from Fig. 4a ($K_1=10^9 \text{ L mol}^{-1}$), to Fig. 4c ($K_1=10^{11} \text{ L mol}^{-1}$), and to Fig. 4e ($K_1=10^{13} \text{ L mol}^{-1}$). In all these cases, as K_3 decreases, the higher is the concentration required to achieve displacement, that is, the higher the dynamic range of the immunosensor, and the lower the analytical sensitivity,

as determined by the change in current for a given concentration of analyte. In the case of a high K_1 , the lower sensitivity is not a result of less antibody bound to the immunosensing interface, but of the greater difficulty in displacing the antibody. At the other extreme, shown in Fig. 4a, a low K_1 results in an immunosensor that is already >60 % unsaturated before any antigen is added, regardless of the K_3 value.

The right-hand side of Fig. 4 shows how variations in K_1 affect the level of unbound epitope as we progress from a low K_3 of 10^8 L mol^{-1} in Fig. 4b to $K_3=10^{11} \text{ L mol}^{-1}$ in Fig. 4d and finally, $K_3=10^{12} \text{ L mol}^{-1}$ in Fig. 4f. Firstly, it is noticed that K_1 needs to be at least in the order of $10^{10} \text{ L mol}^{-1}$ ($\log K_1=10$) to ensure that more than half of the epitope is saturated with an antibody before any antigen is added. Provided this is the case, the dynamic range for the antigen detection can be somewhat be tuned by choosing an appropriate combination of K_1/K_3 values. It is also worth noting that the slope of these responses becomes more gradual the larger the K_1 value is, resulting in a broader dynamic range for these devices.

Experimental results

Loading of the immunosensing interface

To experimentally explore qualitative agreement with the model presented above, electrodes were prepared with six different fluoroquinolones as surface-bound epitopes (Fig. S1), with one antibody, anti-enrofloxacin IgM, and one analyte, enrofloxacin. That is, there is only one value of K_3 explored, as this represents the scenario of how to configure the biosensing interface to give the best performance for a given analyte. Furthermore, the values of K_1 possible are determined by the affinity of the different epitopes for the anti-enrofloxacin IgM antibodies. The loading of the immunosensing interface as a function of antibody for the

Fig. 4 The changes in the level of unbound epitope and current (I) with $[E_x]_0$ and $[Ab]_0$ is fixed at $10^{-9} \text{ mol L}^{-1}$ as a function of $\log[\text{Antigen}]$; **a** as function of $\log K_3$ with $\log K_1 = 9$. **b** As function of $\log K_1$ with $\log K_3 = 8$. **c** As function of $\log K_3$ with $\log K_1 = 11$. **d** As function of $\log K_1$ with $\log K_3 = 11$. **e** As function of $\log K_3$ with $\log K_1 = 13$. **f** As function of $\log K_1$ with $\log K_3 = 12$

six different epitopes is shown in Fig. 5. This is the experimental equivalent to Fig. 2. The IC_{50} (the concentration of antibody required to give 50 % of total current suppression) values obtained are summarized in Table S1. The order of the IC_{50} was enrofloxacin < norfloxacin < ciprofloxacin < enofloxacin < danofloxacin < ofloxacin, and hence, the order of the respective antibody/epitope apparent affinity constant (K_a) was the opposite (Table S1). To show that the decrease in current is caused by antibody binding specifically with the surface-bound epitopes, a control experiment was conducted with biotin as the epitope. As observed in Fig. 5, biotin as the surface-bound epitope results in no significant current attenuation. This indicates that the antibody used in this study is only specific to the detection of fluoroquinolone enrofloxacin and

some of its analogues (i.e., ciprofloxacin, norfloxacin) and that little, or no, nonspecific binding was occurring. Note that we use the terms “apparent affinity” and “apparent affinity constant” here to describe the interactions between an antibody/epitope affinity in a heterogeneous system. This is because the binding curves can only be used as estimates of affinity constants [25] because factors such as ionic strength [26], diffusion limitations, and mass transport [27–29] may hinder the antibody from binding to the surface-bound epitope. The central point of Fig. 5 is that it shows qualitatively similar behavior to Fig. 2 in that, as the affinity of the antibody for the epitope decreases, so does the amount of antibody that binds to the sensing interface, as determined here by the suppression in current. There are however clear differences

Fig. 5 The antibody titration curves obtained for six fluoroquinolone epitopes. The electrode interface was modified with reductive absorption of in situ-generated aryl diazonium cations MW/OEG 1:50. Each data point was the mean value of double determinations from two individual electrodes. The relative standard deviation for five electrodes made in the same manner was 6.8 % [9]. Note that all sigmoidal lines shown were fitted using OriginPro8, function Logistic, and do not represent theoretical fits

as well. As can be seen from Fig. 5, as the epitope affinity for the antibodies decreased, the extent of current suppression also decreased and reached a minimum value that did not coincide with all the surface-bound epitopes being occupied with antibody. That is, all the surface epitopes were never saturated with some of the lower-affinity epitopes. This is in contrast to Fig. 2 where, regardless of K_1 , with high enough antibody concentrations, all surface binding sites are filled. This departure in trend observed for the experimental results compared with the model becomes important below when discussing the change in sensitivity of different displacement assays with different surface epitopes.

Displacement assay

The next question to ask is: How well does the experimental system agree with the model for the displacement assay? To answer this question, the calibration curves for the detection of enrofloxacin using the six different fluoroquinolone epitopes, plus biotin as a control, were obtained via a displacement assay. The amount of anti-enrofloxacin antibody used in all cases henceforth was $0.4 \mu\text{g mL}^{-1}$. In a displacement assay, the analytical signal obtained is directly proportional to the amount of antibody displaced. The three important parameters for a displacement assay are (1) the sensitivity, (2) the limit of detection (LOD) (it is regarded as IC_{10} in this case), and (3) the dynamic range (it is regarded as between IC_{10} and IC_{90}). The calibration plots for enrofloxacin in 50 mmol L^{-1} phosphate buffer (pH 7) for the six different

surface-bound epitopes ($\log_{10}$ concentration) are shown in Fig. 6a.

The impact of the epitopes on the analytical sensitivity can be seen in Fig. 6a where the highest current sensitivity is for the epitope ciprofloxacin. The sensor sensitivity toward the detection of enrofloxacin was determined by the slope within the linear region of the calibration plot of enrofloxacin ($\log_{10}$ concentration). The order obtained was ciprofloxacin > norfloxacin > enrofloxacin > enrofloxacin (see Table S1 for values). This is qualitative agreement with the trend of the theoretically calculated sensitivity where, if the steepest slope is set at 100 %, the relative slopes are ciprofloxacin (100 %) > enrofloxacin (87 %) $\approx$ norfloxacin (86 %) > enrofloxacin (66 %). Note that, in the case of the three high-affinity epitopes, enrofloxacin, norfloxacin, and ciprofloxacin where experimentally it is shown that all surface sites were occupied with antibody, the order of sensitivity follows the sequence of lower- to higher-affinity constant (Figs. 2 and 5) which is of course the same as higher to lower dissociation constants. Although enrofloxacin has a higher dissociation constant, with this antibody, not all surface sites were filled with antibody and hence less can be removed. The antibody displacement from epitopes ofloxacin and danofloxacin did not behave in accordance with a classical thermodynamic equilibrium model as both curves did not fit a sigmoidal shape (Fig. 6b), presumably because some non-ideal binding process occurred [30]. It is possible that, as the antibody has low apparent affinity to these epitopes, the binding interactions might happen in multiple conformation modes and give a range of binding events [31].

The order of the LOD values (Table S1) calculated from the respective sigmoidal fitting curve was enrofloxacin < ciprofloxacin < norfloxacin < enrofloxacin. This is about an

Fig. 6 a Calibration curves obtained via a displacement assay for detecting small-molecule enrofloxacin in phosphate buffer (50 mmol L^{-1} , pH 7). For the comparison of sensor performance, the electrode surfaces were modified with six fluoroquinolone epitopes. The electrode interface was modified with reductive absorption of in situ-generated aryl diazonium cations MW/OEG 1:50. Each data point was the mean value of at least two individual electrodes. Note that all sigmoidal lines shown were fitted using OriginPro8, function Logistic. The surface-bound biotin (a negative control) generated negligible sensor response. The relative standard deviation for five electrodes made in the same manner was 6.8 % [9]; **b** normalised calibration curves for detecting small-molecule enrofloxacin in phosphate buffer (50 mmol L^{-1} , pH 7) by employing different surface-bound epitopes. For the normalization of calibration curve, the highest value obtained via a displacement assay was first set as value 1. The rest of the data were then normalized accordingly. From small to large, according to binding strength: enrofloxacin < ciprofloxacin < norfloxacin < enrofloxacin. Danofloxacin and ofloxacin did not show the classical sigmoidal calibration curve, presumably due to lower binding affinity, and were not considered here. **c** Theoretical calibration curves showing the analogy to Fig. 6a with the experimentally determined K_1 values used in the model

order of magnitude higher than our theoretical values shown in Fig. 6c with the order of LOD somewhat different with enrofloxacin < norfloxacin < ciprofloxacin < enofloxacin. The

dynamic range for each type of sensor with respective surface-bound epitope was regarded as between IC_{10} and IC_{90} . The impact of the epitope on the dynamic range can most easily be observed in Fig. 6b, whereas the calibration curves in Fig. 6a are normalised relative to the maximum change in current for each epitope. The sequence of the dynamic range values (Table S1) obtained was enofloxacin < ciprofloxacin < norfloxacin < enrofloxacin. This order is again consistent with the decreasing apparent affinity constant and higher percentages of dissociation of antibody from the surface.

The experimental results depicted in Fig. 6 show that the best sensor performance, with regard to broad dynamic range and LOD, is using enrofloxacin as the surface epitope, while with regard to sensitivity, ciprofloxacin is the epitope of choice. The advantage of ciprofloxacin arises because, experimentally, this is the lowest-affinity epitope which fulfils the condition of all the surface-bound epitope sites being associated with an antibody. The model illustrates the same general trends and teaches us that higher-affinity antibodies for both the surface-bound epitope and the analyte give superior performance with regard to sensitivity and LOD and that the dynamic range of the immunosensor is observed at lower concentrations for higher values of K_3 and lower values of K_1 .

The one significant departure between the theory and experiment is that the theory predicts that, regardless of the value of K_1 , all surface-bound epitopes will be filled by antibody at high enough antibody concentrations. This was not observed experimentally. Experimentally, it was observed that, as K_1 decreased, so did the extent of saturation of surface-bound epitopes. The result of this less-than-complete saturation was that the highest sensitivity over a broad range of concentrations and the largest change in analytical signal (current in this case) were observed for the combination of ciprofloxacin on the surface and enrofloxacin in solution. In contrast, the model suggests the highest sensitivity is achieved with ciprofloxacin, but the largest change in analytical signal would be achieved with enrofloxacin. Why these differences are observed has not been fully elucidated, but it may relate to some heterogeneity in the surface-bound epitopes that cannot be accounted for in the model.

Conclusions

The impact of the surface epitope and the affinity between this epitope and an antibody binding partner on the performance of immunosensors based on a displacement assay was investigated theoretically and experimentally using an electrochemical immunosensor. In the experimental study, the epitopes used were six fluoroquinolone antibiotics with anti-enrofloxacin IgM as the antibodies and enrofloxacin as

the analyte. The conclusions from the data presented herein are relevant to displacement assays in general. The theoretical and experimental studies show broad agreement with regard to the conclusions. What is shown is that, generally, the higher the affinity of the epitope for the antibody, the more antibodies bind to the surface, but the harder it is to remove. The word “generally” is used here because three of the epitopes, enrofloxacin, norfloxacin, and ciprofloxacin, had sufficiently high affinity for the anti-enrofloxacin antibodies that all surface binding sites were occupied. The number of surface binding sites occupied is important, as the analyte sensitivity of a displacement assay is determined by the number of antibodies that can be dissociated from the surface. In the cases when there is a maximum number of antibodies complexed to the surface, the epitope/antibody combination with the lowest affinity constant gave the highest sensitivity and lowest limit of detection with the dynamic range over which the immunosensor operated being in a lower range of concentration.

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