



The Role of Ligand Rebinding and Facilitated Dissociation on the Characterization of Dissociation Rates by Surface Plasmon Resonance (SPR) and Benchmarking Performance Metrics

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Abstract

Surface plasmon resonance (SPR) is a real-time kinetic measurement principle that can probe the kinetic interactions between ligands and their binding sites, and lies at the backbone of pharmaceutical, biosensing, and biomolecular research. The extraction of dissociation rates from SPR-response signals often relies on several commonly adopted assumptions, one of which is the exponential decay of the dissociation part of the response signal. However, certain conditions, such as high density of binding sites or high concentration fluctuations near the surface as compared to the bulk, can lead to non-exponential decays via ligand rebinding or facilitated dissociation. Consequently, fitting the data with an exponential function can underestimate or overestimate the measured dissociation rates. Here, we describe a set of alternative fit functions that can take such effects into consideration along with plasmonic sensor design principles with key performance metrics, thereby suggesting methods for error-free high-precision extraction of the dissociation rates.

Key words Off-rate, Binding kinetics, Facilitated dissociation, Dissociation, Plasmonic sensors, Surface chemistry

1 Introduction

Surface plasmon resonance (SPR) is a label-free optical method that has been used heavily to characterize the kinetic properties of a wide array of biomolecular systems [1–3], including antigen–antibody interactions, host–guest interactions on molecular cavities, drug affinity, viral activity, protein–DNA kinetics, enzymatic activity, as well as biosensing applications at multiple settings, such as clinics, border security, and point-of-care [4–6]. The fundamental working principle of SPR relies on the alterations of the refractive index (RI) (combining changes in bulk and binding to the surface) of a *reaction volume* right above a surface, on which the amount of

biomaterial varies over time [7]. This mechanism allows the detection of solution-phase ligands associating with the binding sites immobilized on the surface, or inversely, of the dissociation of the ligands off their binding sites into a ligand-free solution in the course of time.

The general methodology of the SPR protocol begins with the loading of the surface-immobilized binding sites with corresponding ligands by flowing a solution containing the ligands over the surface [8, 9]. Once the loading of the binding sites by the ligands is complete, the response signal reaches a saturation level (Fig. 1). The next step is to flow a ligand-free solution that washes off the unbound ligands in the solution. After this step, ideally the setup contains only those ligands that are bound to their binding sites. This state, where lots of bound ligands on the surface with no ligand in the solution, is not energetically favorable (ligands lose translational entropy when they are bound). Thus, the bound ligands begin to leave their binding sites to dissociate into the ligand-free solution until the (average) total free energy change per ligand becomes zero. Since the RI of the reaction volume changes as the ligands diffuse away from the surface, the dissociation of a large number of ligands from their binding sites ideally manifests themselves as a decay phase in the response signal (Fig. 1). This decay continues until fractions of bound and free ligands obey the detailed balance, that is, when chemical equilibrium is reached (Fig. 1).

The half-life of the decay phase of the signal is related to the time scale of the microscopic dissociation. The inverse of this time scale is referred to as the dissociation or off-rate, k_{off} (with the units of inverse time, 1/s). The off-rate provides key information about the frequency of unbinding events, which is of paramount importance to determine the relevant equilibrium association or dissociation (i.e., K_a or K_d) constants of the reaction. Conventionally, the dissociation rate can be extracted by fitting the decay phase of the response signal to an exponential function in the form of

$$f(t) = \exp(-k_{\text{off}}t). \quad (1)$$

The use of the exponential function, Eq. 1, implicitly assumes that the dissociation process of each ligand off its binding site is independent of other ligands in the system. In other words, if one prepares a hypothetical system that contains only a single binding site with a single ligand bound to it, and then, obtain the distribution of dissociation times after *a sufficiently high number* of independent but completely identical experiments, the resulting distribution would be exponential. However, in typical SPR (or in similar single-molecule) setups, the binding sites are often densely placed (e.g., ~1–10 nm) in order to ensure the efficient binding of ligands to the surface during the association phase (Fig. 1). As a

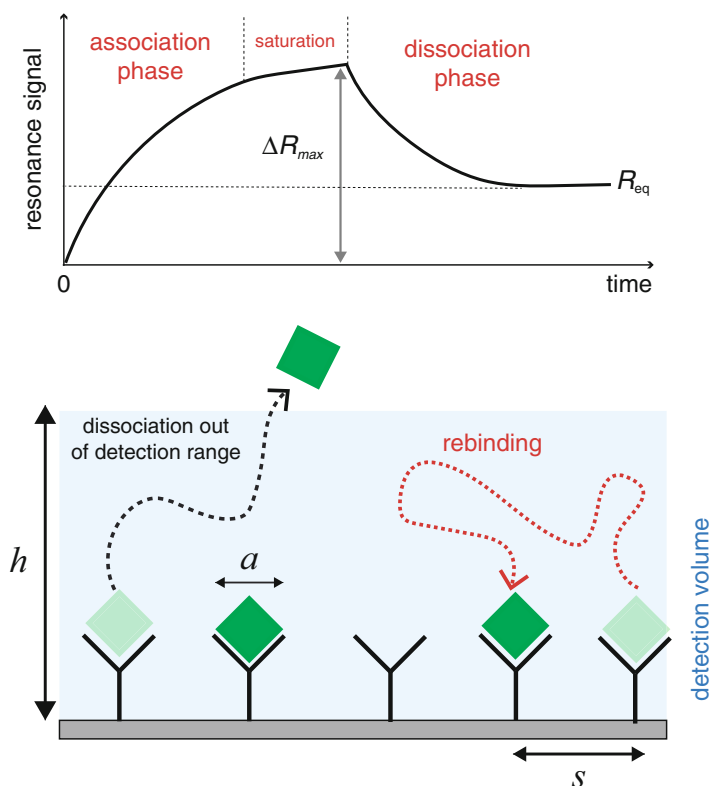


Fig. 1 Upper panel: A typical SPR signal. During the association phase, the binding sites are loaded with ligands until a saturation phase is reached. Then, a ligand-free solution is injected into the system to initiate the dissociation of the bound ligands. Lower panel: During the dissociation phase, a ligand can either diffuse out of the detection volume near the surface with a height of h or rebound to a neighboring empty binding site at a distance, s , thus, delay the dissociation time

result, once the dissociation phase starts, there would be a coexistence of free and bound ligands at any instant; some ligands would leave their binding sites, but they would be still diffusing near the surface. These free ligands can rebound to an empty binding site (including their own) or collide with stably bound nearby host–guest complexes and decrease their stability. In other words, the asynchronous dissociation of ligands could lead to a decay signal that might not be described by an exponential decay. When such data is treated by the exponential fit function (Eq. 1), the extracted value of the off-rate would be lower or higher than the intrinsic value. Most importantly, the usage of Eq. 1 could cause variations even for the same host–guest system from experiment to experiment due to several factors related to system preparation. In this chapter, we discuss two such factors that can significantly influence the entire dissociation process, and thus, argue against the usage of

exponential fits, particularly for weakly binding ligands and densely packed binding sites. We suggest alternative fit functions that can improve the uncertainty in measured reaction rates and possibly decrease the discrepancy between independent measurements along with a discussion on the control of surface chemistry to adjust the distances between recognition elements for ligand binding.

2 Methods

In the following sections, we describe the two effects that can introduce correlations between the unbinding events of molecular ligands (guest) as they leave their binding sites (host). Without going deeply through the mathematical details, the physical mechanism behind these effects will be summarized, and the corresponding corrections to the exponential fit function will be given. Accordingly, we will elaborate the key performance metrics in decoding an efficient sensing feature through material types of plasmonic sensors and surface chemistry approaches and minimizing the external effect to maintain direct host–guest interactions. Consequently, our ultimate goal is to guide researchers from various scientific backgrounds to use these fit functions with minimal effort to improve the measurement quality in their kinetic experiments or molecular dynamics (MD) simulations. We also aim to guide them to choose the most appropriate options for sensor hardware, soft biological layers, and maintaining high-selectivity before using plasmonic platforms.

2.1 Rebinding of Ligands Due to High Surface Density of Binding Sites

SPR principle relies on the dissociation of a large number of ligands from their binding sites into a ligand-free reservoir. To increase the measurement precision, a large number of surface-immobilized binding sites are often needed. This inevitably results in a high grafting density of binding sites on the surface and a reduction of the average distance between the sites. We may estimate the average distance between binding sites, s , via calculating the amount ligands on the surface as follows:

$$s \approx 1.5 \left(\frac{M_w}{\Delta R_{\max}} \right)^{1/2} \text{ nm},$$

where M_w is the molecular weight of ligands in the units of Dalton, and $\Delta R_{\max}$ is the maximum signal in the units of resonance units (RU) (Fig. 1). For a typical saturation value in the SPR signal of $\Delta R \approx 100$ RU (1 RU = 1 pg/mm² as reported in the technical features of the Biacore System [10]), the average distance between two binding sites is $s \approx 15$ nm if we assume a molecular weight of 10 kDa (*see Note 1*). Notice that the grafting density of binding sites is defined as $\sigma_g = 1/s^2$, thus the equation above can also be used to obtain the average grafting density of binding sites.

While a high grafting density can improve the statistics of the dissociation phase, a ligand, upon its unbinding, can rebound to its own or another nearby empty binding site rather than exiting outside of the detection volume (Fig. 1). Those ligands would not contribute to the dissociation signal even though they have evacuated their original binding sites since the fraction of ligands within the evanescent field (reaction volume) does not change. A quantitative estimate can be made for the time scale of the rebounding events. On average, a dissociated ligand can diffuse to neighboring binding sites located at a distance s after a diffusion time of $\tau_s = s^2/4D$, where D is the self-diffusion coefficient of the ligand [11, 12]. If the distance between the neighboring binding sites, s , is much larger than the height of detection volume (i.e., roughly the decay length of the evanescent field, $h \approx 100$ nm) [10], the dissociating ligands would have a higher probability of diffusing away from the surface and a low probability of rebounding. However, if the binding sites are placed densely (i.e., $s \ll h$), the ligands could diffuse to nearby empty binding sites before exiting the detection volume. Those ligands would exit the detection volume eventually but only after spending a certain amount of time at other binding site(s). As a result, the average residence time of the ligand within the detection volume would not reflect the actual microscopic dissociation time of the ligands off its binding site but rather provide a value about the total dwell time of the ligands near the surface (inside the detection volume).

Several MD and Monte Carlo (MC) simulation studies on model systems as well as experiments on insulin-like growth factors interacting with their binding proteins [11] systematically demonstrated that the effect of rebounding on dissociation rates is readily observable for densely placed binding sites [11–14]. To extract the intrinsic off-rates (unaffected by rebounding events) from the response signal, an analytical expression that takes the rebounding events into account has been suggested in the form of a *stretched*-exponential fit function as [11]:

$$f(t) = \exp\left(\frac{k_{\text{off}}^2 t}{k_s}\right) \text{erfc}\left(\sqrt{\frac{k_{\text{off}}^2 t}{k_s}}\right). \quad (2)$$

In Eq. 2, $k_s = 1/\tau_s = 4D/s^2 = 4D\sigma_g$ is the rate characterizing the rebounding events, and $\text{erfc}(x)$ is the error function. The key point in Eq. 2 is that as k_s approaches to k_{off} (i.e., as the separation distance between binding sites increases), and Eq. 2 turns into the conventional exponential function given by Eq. 1. In short, Eq. 2 suggests a correction to the off-rates, which mainly increases its effect for densely packed binding sites (*see Note 2*).

MD simulations can directly demonstrate the effect of rebounding occurrences on off-rate measurements (Fig. 2). Since MD can track the trajectories of individual ligands to determine their

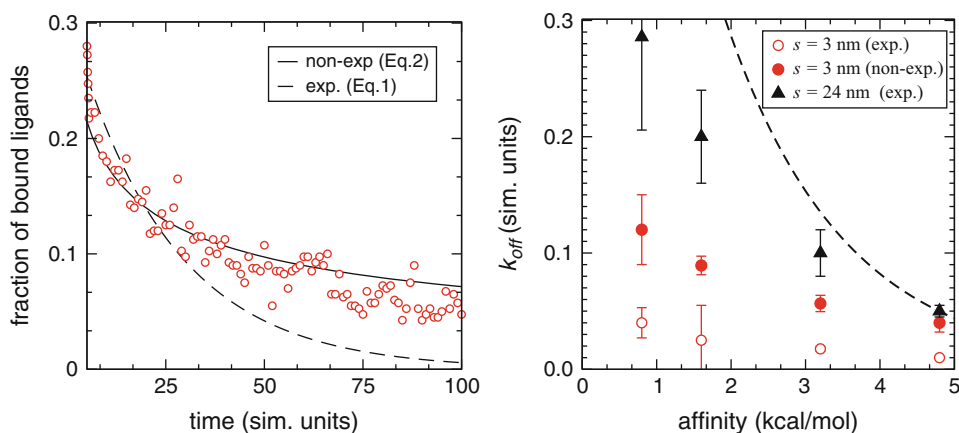


Fig. 2 MD simulations of monomeric ligands dissociating from their binding sites. Left panel: The fraction of bound ligands decreases as time progresses. The time is given in simulation units. Exponential (dashed curve) and the non-exponential (solid curve) fits to the dissociation data are plotted by releasing the k_{off} as a fit parameter. Right panel: The off-rates obtained from the fit procedure for various binding affinities by using both methods. In all cases, the off-rates decrease with increasing affinity. As the average distance, s , is increased, the off-rate of the ligand approaches the Arrhenius limit ($k_{\text{off}} \sim \exp(-\text{affinity})$). (Adapted from Ref. 12 by assuming a simulation unit length scale of $a = 1$ nm)

binding states, they can easily interrogate a range of separation distances and binding affinities. Figure 2 shows the data obtained from a set of such MD simulations [12] with spherical ligand particles of size $a \approx 1$ nm that are initially immobilized on their binding sites. Thus, the simulation in that study specifically models the beginning of the dissociation phase, at which all binding sites are already loaded by ligands (Fig. 1). The ligands interact with their binding sites weakly with affinities ranging between $E \approx 1$ kcal/mol and 5 kcal/mol, which are typical numbers for small analytes or proteins interacting with their receptors [15]. In those computational experiments, binding sites are placed on the surface with an average distance of $s = 3$ nm; at this short separation distance, we expect to see a considerable effect of rebinding events on the dissociation kinetics. Indeed, if the dissociation data is fitted both to an exponential function (Eq. 1) and to the alternative fitting equation (Eq. 2) separately, the exponential fit gives a ten times slower off-rates as compared to the non-exponential fit in Eq. 2 (Fig. 2). If the same experiments are repeated by sparsely placing the binding site with $s = 24$ nm, thus bringing the value of k_s closer to k_{off} , the extracted off-rates for exactly the same host–guest pairs are closer to the k_{off} value extracted via Eq. 2 (Fig. 2). This finding suggests that if the binding sites are sparsely placed, the effect of the rebinding events on off-rates can be reduced significantly. When simulations are repeated with higher affinities, the off-rates obtained by Eq. 2 converge better to the off-rates obtained by sparsely placed binding sites, but no improvement is

observed for the conventional exponential fit (Fig. 2). These simulations readily demonstrate the critical effect of the binding-site placement on the dissociation kinetics of molecular ligands (e.g., drugs, proteins) and in the meantime validate the use of the non-exponential fit functions given in Eq. 2 in the extraction process of off-rates for any SPR setup with arbitrary surface coverage.

2.2 Facilitated Dissociation (FD) of Ligands by Solution-Phase Ligands

During the last decade, a large number of experimental and computational studies have uncovered a concentration-dependent dissociation mechanism for various biomolecular ligands [16]. Particularly, small DNA binding proteins (e.g., bacterial transcription factors) [17] as well as subunits of large protein complexes (DNA polymerase) [18] exhibit a dramatic increase in their dissociation rates at the sub-micromolar concentrations levels. Furthermore, MD simulations inspired by single-molecule experiments demonstrated that this effect can be operative in SPR-like setups [19]. Such a stoichiometry-dependent mechanism is quite surprising and unexpected since the classical textbook view assumes that the dissociation rate has no concentration dependence [20].

The underlying mechanism for the concentration-dependent dissociation kinetics is attributed to the multivalent binding nature of molecular ligands, which allows bound ligands to partially expose the binding sites for other binding competitors in solution [21]. Indeed, many molecular ligands are bound to their binding sites with such multivalent interactions. For instance, proteins bind to their receptor or to DNA via a combination of hydrogen bonds, and electrostatic and van der Waals interactions. A bound ligand such as a nucleic acid-binding protein or a drug molecule can *break* some but not all of these multivalent interactions and easily expose the binding site for the binding competitors in solution. The resulting presumably less stable complex of more than one ligand on a single binding site can promote rapid unbinding of both or one of the bound ligands. This effect is referred to as facilitated dissociation (FD) or exchange, and it is mathematically described as follows [22]:

$$k'_{\text{off}} = k_{\text{off}} + k_{\text{exch}}[c] \quad (3)$$

The expression given by Eq. 3 suggests that as the free ligand concentration in solution, c , increases, the off-rate accelerates linearly with the concentration. The correction in Eq. 3 operates only when the ligand concentration is non-zero (i.e., when $c = 0$, $k'_{\text{off}} = k_{\text{off}}$). In Eq. 3, the exchange rate, k_{exch} , has the units of $\text{M}^{-1} \text{s}^{-1}$ and determines the strength of the facilitated dissociation effect. The typical values of $k_{\text{exch}} \approx 10^4 \text{ M}^{-1} \text{s}^{-1}$ for mid-size ($\sim 5 \text{ nm}$) transcription factor proteins unbinding from dsDNA at physiological conditions [17].

In a typical SPR setup, FD can alter the intrinsic dissociation rates as follows. During the dissociation phase, free ligands (recently dissociated) and bound ligands can coexist. As a result, the free ligands can collide with the intact host–guest complexes and accelerate their dissociation via a direct FD as described by Eq. 3. FD could be more influential at the beginning of the dissociation phase due to the non-uniform distribution of the ligands inside the measurement reservoir. This is mainly because, at the beginning of the dissociation phase, more ligands are located near the surface than within the bulk due to the formation of a concentration gradient [12]. In equilibrium, this concentration gradient ideally would disappear, and the ligand concentration in the reservoir would become homogenous. However, at early and intermediate times (close to the intrinsic residence time of the ligand on the binding site), the time-dependent relaxation of the initial concentration quench could lead to FD.

Unlike rebinding, an analytical expression that can correct the side effects of FD on off-rates, as in Eq. 2, does not exist. This is mainly because such expression is expected to depend on the unbinding energy (thus, on the off-rate itself) for the reason that we describe above. However, since the spread of free ligands throughout the reservoir is mainly governed by diffusion, an expression for the time-dependent concentration difference between bulk and near-surface, $\Delta c(t)$, may be written as follows. The average distance that the ligands can diffuse in the vertical direction at time t is Z , with $Z^2 = 2Dt$, where D is the diffusion coefficient of the ligand. If the total number of ligands in the setup (on the surface at the beginning of the dissociation phase) is N , then the concentration of ligands inside a volume, whose height is determined by the diffused distance Z , may be written as $\Delta c(t) \sim N/A \times Z = \sigma_g/(2Dt)^{1/2}$, where A is the area of the reaction surface, and $\sigma_g = N/A = 1/s^2$ is grafting density. In this case, the concentration difference is simplified to

$$\Delta c(t) \frac{\tilde{1}}{s^2 \sqrt{t}}. \quad (4)$$

Equation 4 suggests that, for large period of time, $\Delta c(t) \rightarrow 0$, and consequently the concentration difference throughout the reservoir becomes uniform. For large separation distances, $s \gg h$, Eq. 4 also converges to zero. This indicates that at least the effect of FD on off-rates could be reduced by preparing sparsely immobilized binding sites. Indeed, in the fluorescence studies of transcription factors, the binding sites that are separated by roughly 1 micron apart were shown to lead to exponential decays [16]. This is mainly because FD, as in the rebinding case that we discuss above, also requires the diffusion of free ligands to neighboring binding sites. However, unlike in the rebinding effect

(which requires a neighboring binding site to be empty), FD affects only those binding sites that are occupied by ligands.

2.3 Deciphering the Key Performance Metrics of Plasmonic Sensing

Common strategies on plasmonic sensors rely on (1) sensitivity within the detection volume (Fig. 1), where the resonance angle or wavelength shifts according to RI changes; (2) thin-film sensitivity, which is caused by the loaded mass, resulting in resonance shifts proportional to the changes in the film thickness; and (3) figure-of-merit (FOM) is the ratio of resonance shifts with linewidth [23]. As elaborated in the earlier subsections, the SPR sensing scheme is considered as a gold standard method to evaluate binding kinetics and estimate interactions between ligands and their binding sites reliant on the adsorption isotherm models (Scatchard, Langmuir, and Freundlich) as follows [24]:

Master equation:

$$\frac{d\Delta R}{dt} = k_c(\Delta R_{\max} - \Delta R) - k_{\text{off}}\Delta R \quad (5)$$

Scatchard:

$$\frac{\Delta R_{\text{ex}}}{c} = K_a(\Delta R_{\max} - \Delta R_{\text{eq}}) \quad (6)$$

Langmuir:

$$\Delta R = \frac{\Delta R_{\max}[c]}{K_d + [c]} \quad (7)$$

Freundlich:

$$\Delta R = \Delta R_{\max}[c]^{1/n} \quad (8)$$

where ΔR is the binding response; k_c and k_{off} are respective on and off-rates with units of 1/s. In Scatchard and Langmuir isotherms, K_a and K_d are also the forward and reverse equilibrium constants; subscripts *ex*, *max*, and *eq* indicate experimental, maximum, and equilibrium, respectively. The exponent $1/n$ is known as the Freundlich exponent. Scatchard isotherm exhibits freely reversible host-guest interactions and denotes the number of binding sites once the host is at equilibrium [25, 26]. Langmuir isotherm model states a homogeneous binding occurred on the sensor surface [27, 28], whereas the Freundlich model displays a heterogeneous model, being favorable with the low concentration regime and elucidating multilayer formation on the surface [29].

While considering the detection limits, specificity, and sensitivity, there are the other metrics impacting the performance of sensor that include hardware (e.g., sensor structure/material, detector, light source, and optical path), soft biological layer (surface chemistry), as well as external factors (RI and complexity of solution). Nanoplasmonic sensors, for instance, denote tunable fashions through probing more binding sensitivity and exhibiting the

reduced sensing in the bulk. Upgraded sensors that are mostly integrated with advanced materials like graphene or designed with well-confined nanostructures provide sharper resonance signals than that of conventional SPR systems, enabling easy probing the resonance changes in minute concentrations of the sample [30]. In the following subsections, we comprehensively reckon all inherent factors affecting key performance metrics, i.e., material types for sensor (hardware), surface functionalization (soft biological layer), and antifouling strategies (for minimizing external factors) that can be used to reduce the side effects in the measurements that we describe in the previous sections.

Recent advances in materials science have empowered the performance and versatility of plasmonic sensors by integrating new-fangled materials into sensor manufacturing [31]. The sensors are mainly fabricated via (1) top-down approaches to create nanostructures or produce metamaterials and hyperbolic surfaces, and alternatively and/or via (2) bottom-up approaches to self-assemble nanoparticles with plasmonic features. Before considering any chemical approaches, material type (gold, silver, aluminum, and palladium) and composition profoundly impact on sensing performance since surface property defines the choice of chemistry to properly sense target analytes.

2.3.1 Surface Functionalization

The sensor surface consists of two major areas: (1) analyte-specific unit (bioactive area), where recognition elements (binding sites) are localized to provide specific host–guest interactions; and (2) inert area that is blocked for non-specific interactions (i.e., phantom binding sites). Signals derived from the surface is usually proportional to the yield of binding events influenced by the surface density of binding sites and the specificity between this area and inert area [32]. In this regard, the specificity of target recognition, the orientation of immobilized binding sites, and the selectivity of the surface coverage (the integration of spatial ratio of bioactive and inert areas addressed by antifouling agents) are three major strategies for designing surface chemistry on the sensor systems [31].

The “specificity” parameter relies on the affinity of molecules (binding sites and ligands), being proportional to a function of the number of both physical and chemical interactions between them. The first interaction type consists of the immobilization of binding sites without any disruption or changes in the native conformation [32]. The latter interactions are the combination of weak molecular interactions between ligands and binding sites. Since these interactions play pivotal signals for the kinetic systems, the analytes are mostly captured on the surface by a bioactive unit, and also labeled with another bioactive unit in order to leverage the specificity of the sensor [33]. The recognition elements on the bioactive area are mostly chosen within the families of immunoglobulins (Ig) or antibodies, in particular, from the IgG family. They are in the

glycoprotein structure and consist of two major polypeptide chains: heavy ($-\text{COOH}$ end) and light chains ($-\text{NH}_2$ end). Briefly, two heavy chains form fragment crystallizable (Fc) region of an antibody (constant part), whereas two light chains exhibit fragment antigen-binding (Fab) region of an antibody for recognition, thereby acting as a crucial spot for specific recognition. Besides, aptamers, other commonly employed bioactive units, are rationally designed on nucleic acid format, but acting as antibody-type recognition. They are however produced through lengthy and expensive procedures, limiting their expansion to the field. Among these units, nucleic acids, carbohydrates, and molecularly imprinted polymers are utilized [34]. Regardless of the structure and chemical composition of binding sites, the most essential step in the surface chemistry is to orient these units in the right direction to capture the target ligands properly. The “orientation” of binding sites holds two major roles: (1) properly oriented units exhibit stronger efficiency for target analyte recognition, as well as minimize any non-specific interactions [33, 35]; and (2) highly oriented units enable high recognition and specificity, increasing sensor sensitivity and maximizing the sensor performance. Oriented self-assembled coupling (chemisorption), covalent coupling with chemical linkers, and bioaffinity coupling reactions are usually projected for this manner.

Surfaces coated with Ag or Au allow weak interactions with antibodies and also allow forming dative bonds via disulfide bridges originated from cysteine residues of antibodies or thiol groups of aptamers [32, 36, 37]. Despite easy application of chemisorption, the orientation and the specificity cannot be controlled since this process is random, and most likely, metal-reactive groups of other biomolecules might bind to both the active and inert areas of the sensor. As multiple washing steps are performed during the experimentation, such weak interactions might be liberated from the surface. In such cases, antibodies could be spliced into two fragments with a reducing agent (2-mercaptoethylamine (2-MEA), tris (2-carboxyethyl)phosphine (TCEP), 2-mercaptoethanol (2-ME), or 1,4-dithiotreitol (DTT)) that reacts with disulfide bond of Fc portion, allowing the fragments to be bound via thiol groups to the metal surface, and also ensuring to preserve the right orientation [31, 38]. Despite being easily applicable, this strategy requires strictly controlled conditions to keep the antibody structure native and to maximize the surface coverage (i.e., grafting density); hence, such a strategy could be a better option for immobilizing aptamers that are easily engineered according to this scenario.

Covalent coupling with chemical linkers is one of the most commonly employed strategies during the activation and immobilization of binding sites on the sensors. This process not only improves the stability but also creates a well-harmonized structure by anchoring a bifunctional self-assembled monolayer (SAM) (one

binds to the metal surface and the latter acts as a linker), whose linker-end complements with bioactive unit molecules. The chain size and functionality of SAM molecules are chosen according to the reactive groups of bioactive units. For this process, in-solution method depends on multiple parameters, e.g., pH, temperature, local roughness, crystal orientation, and oxidation of the metal surface [31]. As an example, a gentle washing with piranha solution or treating with oxygen plasma helps oxidation on the surface that improves the strength of the S–Au bonds while forming SAM [39]. Vapor functionalization—another strategy for covalent coupling—is carried out at ambient pressure [40] or under high-vacuum conditions in order to achieve well-ordered monolayers, yet the utility of this method is limited due to the need for complex setups and multistep procedures [41].

The latter strategy, bioaffinity coupling, secures the oriented immobilization, reliant on weak interactions between the linker and the recognition element that allow a highly specific and strong recognition mechanism. Avidin–biotin interaction is a commonly used strategy exhibiting extremely high affinity with a dissociation constant $K_d \approx 10^{-14}$ mol/L. In practice, avidin molecules are immobilized on the surface, while biotin molecules are linked to the recognition elements (mostly an antibody, nucleic acid, or aptamer). The order of these molecules might be vice versa. However, constructing the right orientation relies on the position and number of biotin molecules on the recognition elements. Another bioaffinity coupling is applied via antibody-binding proteins such as Protein A and Protein G that selectively bind to the Fc portion of antibodies, guiding them to be anchored in the correct orientation [42].

2.3.2 Antifouling Strategies

Ideally, the binding sites are immobilized on the sensing area, whereas all the surrounding needs to be inert for any kind of non-specific interactions. Forming inert areas have pivotal roles in developing high sensitivity capable of detecting a drastically low number of target of interest from complex biospecimens (e.g., whole blood, serum, urine, sweat, lavage fluid, and saliva) made of highly dense media that results in high RI changes during the measurement by a conventional SPR platform. When this is a case, constructing an antifouling layer help passivating the non-active area, minimizing or ideally avoiding any nonspecific interactions. Protein-stemmed strategies, such as applying bovine serum albumin (BSA) or casein, are usually preferred for this aim because of their high abundance and easy binding through physio-adsorption. As described above, passive adsorption of molecules could be liberated typically during the multistep washing. Chemical binding of polymeric antifouling agents is thereby preferred to ensure stable conjugation and form flexibility like observed in cell membrane systems [43]. Zwitterionic polymers and poly(ethylene glycol)

(PEG) are the most employed antifouling agents, owing to exhibiting strong surface hydration [44, 45]. While zwitterionic polymers are used, the hydration layer is created by electrostatic-induced hydrogen bonding. On the other side, this layer is formed by hydrogen bonding between water and the ether oxygen atoms while PEG is immobilized on the surface [45–47]. Theoretically, this hydration layer creates a physical and energy barrier, where water molecules cannot be replaced by any biomolecule or larger entities like cells. In some cases, the surface roughness forms superhydrophobic surfaces that aid target molecules to be captured, whereas the adsorption of other molecules in solution is eliminated [45]. Besides, many printing strategies are employed to improve selectivity in sensors by (1) conventional direct printing via an automatized inked tips to form either continuous or discrete areas on the sensor; (2) noncontact printing (NCP) reliant on lithographic stamps, mostly made of PDMS, that transfers biomolecules onto the bioactive area once they are immersed in an inking solution [48]. Nano-stencil lithography (NSL) is another strategy to bring bio-reactive layer in contact with biomolecules through a stencil mask consisting of sub-100-nm patterns [49]. The mask employed in this strategy could be reused multiple times, and it is able to pattern large superficial areas rapidly [49].

3 Final Remarks

In this chapter, we described two effects that can influence the measured dissociation rates by SPR or in general similar setup relying on kinetic mass changes. The first effect arises from the rebinding of ligands to binding sites before exiting the detection volume, thus, adds a delay on the dissociation data. This delay results in an underestimation of off-rates (Fig. 2). The second effect is due to accelerated dissociation of bound ligands by free ligands near the binding site (FD). FD in principle leads to higher off-rates. As we discussed, both effects diminish as the average distance between the binding sites increases (or at low grafting densities). When the measurements are performed for strongly bound ligands (e.g., >5 kcal/mol), the alternative fit function, Eq. 2 (e.g., the stretched exponential [11]), can correct the rebinding effects. However, for weakly binding small ligands (<3 – 4 kcal/mol), even the improved fit function underestimates the off-rates (Fig. 2). Thus, sparsely placed binding sites seem to be the best option to extract the dissociation rates of ligands with very weak affinity in both experiments and computer simulations of SPR systems. The large separation will also reduce the unwanted effect of FD.

Considering the fundamental and experimental interfaces of SPR-type sensors, material type holds a pivotal characteristic on the sensitivity along with the integration of optical setup and provides an opportunity to choose more surface-sensitive or bulk-sensitive measurements [50]. In our calculations, we think that enhancing the sensitivity layer at the vicinity of the sensor will help us monitoring more interactions (especially diffusion-controlled reactions) between target binding sites and ligands. Besides, the surface chemistry and antifouling strategies will enable to tune surface grafting. For instance, polymer-brush type of agents will increase surface area, potentially forming more binding sites and dynamic structure compared to the SAM-based backbone structures (particularly rigid constructions). At the same time, integrating an antifouling layer to the sensor surface potentially minimizes nonspecific binding and increases the direct host–guest interactions during the measurements, thereby enabling more secure data collection to understand the binding, dissociation, and diffusion events. Furthermore, the separation distance between the binding sites could be achieved using a specific ratio of antifouling agents and recognition elements [51].

4 Notes

1. In Biocore systems, 1 RU corresponds to 1 pg/mm², whereas in other systems (Autolab) 0.01 ng/mm² corresponds to 1 m° (millidegree). Hence, the conversion from RU to m° is 1 RU = 0.1 m°.
2. Once the raw data is obtained in (time vs. signal) format, by using excel or other available mathematical manipulation software (e.g., MATLAB, Mathematica), the data can be fit to the function, $f(t)$ in Eq. 2. For instance, in Mathematica, the signal can be read in a datafile with the command “datafile=**Import** [“signal”, “Table”]”. Then, this array can be fit by using the command “**FindFit**[datafile, $f(t)$, k_{off} , t]” to extract the off-rate.

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