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Fixed charges in the gel matrix of sensor chips and dissociation in diffusion gradients influence the detection of fast protein–protein interactions

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ABSTRACT

In molecular interaction studies based on surface plasmon resonance (SPR) measurements, the ligand is often immobilized in a thin carboxydextran gel matrix. Here we investigated the influence of the charged gel on the results of such SPR measurements. At physiological ionic strength, analytes with a net charge of more than about 5 are considerably enriched or depleted due to the Donnan potential under commonly applied experimental conditions. Below physiological ionic strength, enrichment was found to be even stronger than predicted by Donnan theory. The influence of the gel matrix on the apparent binding is prevented in competition experiments, in which SPR measurements are only used to discriminate between free and complexed analyte while the interaction between analyte and ligand is studied in solution. However, if the analyte–ligand interaction is very fast, thermodynamic equilibrium is disturbed near the interface where free analyte binds to the immobilized ligand due to mass transport limitation. Consequently, the soluble analyte–ligand complex dissociates, which results in an overestimation of free analyte. In experiments of calmodulin binding to fragments of the KCNH1 ion channel protein this mass-transport-induced dissociation led to a systematic underestimation of the affinity. We conclude that the insufficient discrimination between the true analyte–ligand binding and the complex interactions of the analyte with the gel phase may result in systematic errors. The theoretical framework for recognizing and avoiding such errors is provided.

1. Introduction

Real-time interaction measurement with surface plasmon resonance (SPR) sensors is a powerful approach to study protein–protein interactions (Rich and Myszka, 2008). One of the proteins (ligand) is often immobilized in a matrix of carboxymethylated dextran that covers the sensor surface, and the interaction partner (analyte) is applied in a laminar flow of buffer along this surface (Johnsson et al., 1991; Sjolander and Urbaniczky, 1991). The standard procedure in BIACORE technology to covalently immobilize a protein ligand to an activated sensor chip surface uses electrostatic interactions. The ligand is applied in a low ionic strength buffer at pH well below its isoelectric point. Under these conditions it carries multiple positive charges, and the electrostatic interaction with the negative charges of COO^- groups in the gel leads to local ligand concentrations several orders of magnitude higher than bulk concentration (Johnsson et al., 1991). Besides to increase the local protein concentration, the carboxy groups of the matrix serve as attachment points for activated groups that allow covalent coupling of the ligands. At the end of the protein immobilization procedure, the reactivity of the activated groups is blocked with ethanolamine. However, residual COO^- groups remain in the matrix after ligand immobilization such that electrostatic interactions between charged ligands and the matrix persist, but they become smaller at higher ionic strength. Although the use of carboxymethylated dextran sensors has become the most popular tool to characterize protein–protein interactions (Rich and Myszka, 2011), the potential influence of such non-specific electrostatic interactions is mostly ignored.

Various scenarios of how the analyte interacts with the immobilized ligand are illustrated in Scheme 1. In (A) the BIACORE signal is generated by binding of the analyte in the gel phase to the immobilized ligand while (B) illustrates the situation when the transport of the analyte through the interface is rate limiting. To avoid systematic errors due to non-specific interactions with the gel matrix, the ligand–analyte binding can be characterized in a competition

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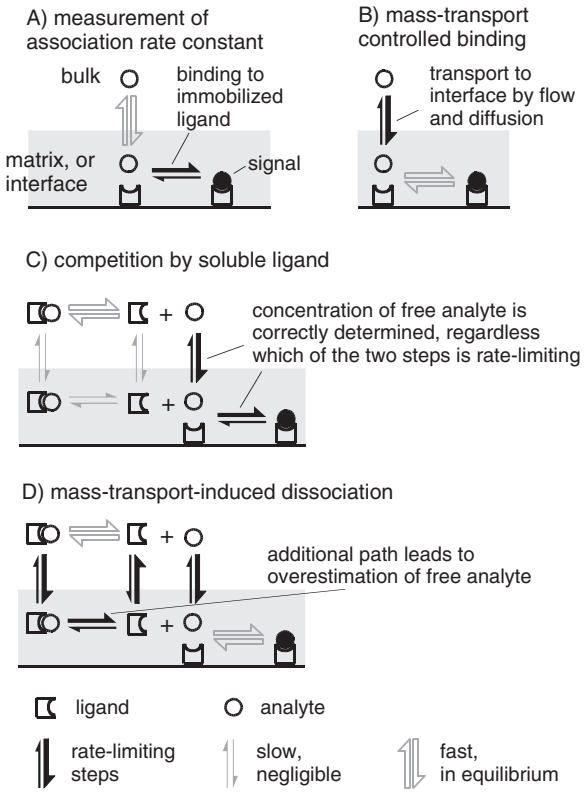

Scheme 1. Scenarios of binding and diffusion processes taking place in a gel matrix of an SPR sensor chip.

assay. As illustrated in Scheme 1C, the interaction of interest takes place in bulk solution. In such a setting reliable equilibrium dissociation constants (K_D) are expected because BIACORE technology is only used to quantify the concentration of free analyte that is still binding to the ligand immobilized on the sensor (Ward et al., 1995; Nieba et al., 1996). Nevertheless, as shown in Scheme 1D, rapid dissociation of the analyte–ligand complex can also disturb this competition assay. The competition experiment (C) measures the concentration of free analyte by comparison with the signal of known analyte concentrations. The rate-limiting step may be the binding to the immobilized ligand (A), diffusion to the sensor surface (B), or a combination of both. Under mass-transport limitation (B), the concentration of free analyte in a thin layer above the sensor surface is reduced due to binding to immobilized ligand. Thermodynamic equilibrium is thus disturbed and the soluble analyte–ligand complex can dissociate. We refer to this effect as "*mass-transport-induced dissociation*" (D). By numerical simulation we studied how charged protein distributes between matrix and bulk solution and investigated under which conditions mass-transport-induced dissociation can influence competition measurements.

To experimentally characterize this phenomenon, we studied the interaction of the Ca^{2+}-binding protein calmodulin (CaM), which harbors an effective net charge of $z = -16$ (Andre et al., 2004), with fractions of the KCNH1 K^+ channel protein, which was previously shown to bind CaM in a Ca^{2+}-dependent manner (Schönherr et al., 2000; Ziechner et al., 2006). We used the Donnan theory (Donnan, 1924) to predict the distribution of charged analyte between matrix and bulk solution, and compared the predictions with measurements of a charged model protein, lysozyme.

Since conformational fluctuations of CaM as well as its target proteins were proposed to play an important role in the interaction (Evenäs et al., 1999; Yamniuk and Vogel, 2004), we finally considered whether binding experiments provide information about the percentage of binding molecules and about the dynamics of possible changes between binding and non-binding states.

2. Materials and methods

2.1. Simulation of BIACORE measurements

Simulations were performed with the program BIA_SIM (Glaser, 1993) (available under the GNU General Public License from http://www.uni-jena.de/~b1glra/bia_sim/), which numerically calculates concentrations of the interacting molecules in a two-dimensional cross-section through a BIACORE microflow chamber. Analogous to an SPR measurement it calculates the amount of analyte bound to immobilized ligand in a thin layer at the interface between analyte solution and sensor surface. The hydrodynamic flow parallel and the diffusion of molecules perpendicular to this interface were considered, with both effects accounting for the transport of analyte to the immobilized ligand. Simulations were run with (BIA_SIM Version 5) and without (Version 4) explicit consideration of the thickness of the gel layer. Numerical simulations were performed with a grid size of 11 (length) × 16 (height). To confirm convergence of the numerical procedure, several simulations were repeated with a grid size of 22 × 32 whereby no significant differences were noticed.

2.2. Distribution of analyte molecules

The distribution of charged analyte molecules between bulk solution and the gel matrix with fixed charges was calculated according to the Donnan theory (Donnan, 1924). We treated the matrix as a uniform compartment with a density of immobilized charges, c_0. The concentration in the matrix of all other ionic species (c_i^m, $i = 1, \ldots, n$) with charge z_i depends on the Donnan potential ψ_D:

$$\frac{c_i^m}{c_i^b} = \exp\left(\frac{-z_i e_0 \psi_D}{kT}\right), \quad (1)$$

where c^b is the concentration in bulk solution, and e_0, k, and T have their usual meaning. As the thickness of the matrix is large compared to the Debye–Hückel length, the matrix can be considered neutral

$$c_0 + \Sigma z_i c_i^m = 0 \quad (2)$$

The simultaneous solution of Eqs. (1) and (2) provides the enrichment factor for the injected analyte, $F = c^m_{\text{analyte}}/c^b_{\text{analyte}}$.

The charge density (η_q) of the carboxylated dextran gel matrix was calculated from the SPR signal of lysozyme, extrapolated to zero ionic strength ($R_{I\to 0}$) from

$$R_{I\to 0} = \text{RP} \times \text{MW} \times \frac{\eta_q}{z}, \quad (3)$$

where $\text{RP} \approx 1\,\text{RU}/10^{-9}\,\text{kg/m}^2$ is the specific SPR signal of proteins (Stenberg et al., 1991), MW = 14.3 kg/mol is the molecular weight of lysozyme, and $z = 8$ is the net charge. This is a fair assumption because at very low ionic strength the solution to Eqs. (1) and (2) shows that the negative charges of the gel matrix are neutralized to nearly 100% by the accumulation of the cationic protein irrespective of its bulk concentration.

2.3. Real-time interaction measurement

Measurements with SPR detection were performed with a BIACORE 2000 and CM5 sensor chips (Biacore AB, www.biacore.com). Due to its low isoelectric point, CaM was enriched for immobilization on the sensor surface in 2 mM citric acid. Measurements were