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Calibration free concentration analysis by surface plasmon resonance in a capture mode

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ABSTRACT

Surface plasmon resonance (SPR) is the gold standard for determining rate and equilibrium constants of bimolecular complexes. Accuracy of these parameters depends on the correct determination of the concentration of the injected analyte. Calibration free concentration analysis (CFCA) has been developed to overcome the limitation of measuring protein concentrations spectroscopically, which may overestimate the fraction of the protein that really binds to the immobilized ligand, *i.e.* the active concentration. In this work, we demonstrate that CFCA can also be implemented in a capture format for measuring active concentrations. Capture CFCA (CCFCA) was first validated by measuring the concentration of a HLA-B*44:02 antigen solution. The active concentration of this molecule determined by CCFCA was similar to that obtained by covalent CFCA. CCFCA was then used to determine the concentration of the W6/32 pan class I HLA monoclonal antibody over three different HLA molecules captured by another specific antibody. This could not have been performed by covalent CFCA because immobilized HLA molecules cannot withstand regeneration. By exploring different capture levels we also show that CCFCA gives consistent results even at low capture levels. Knowing the active concentration of W6/32, we then determined the rate and equilibrium constants of W6/32-HLA complexes on the same flow cell. CCFCA is of general use for measuring active concentrations and of great interest for analytes recognizing ligands that cannot be covalently immobilized on sensor chips. The capture mode also allows determining the kinetic constants of multiple analyte-ligand complexes on the same flow cell. This increases experiments throughput and reduces sensor chip consumption.

1. Introduction

Biosensors based on the surface plasmon resonance (SPR) principle monitor in real time complexes formed between two partners, the ligand being immobilized at a liquid–solid interface on a sensor chip, and the analyte being injected in a continuous flow of buffer [1,2]. This technology has become the gold standard for determining the characteristics of antigen-antibody interactions, especially the affinity of antibodies for their antigenic target [3–5]. The dissociation equilibrium constant, K_D, is determined either by steady-state analysis or kinetically, by measuring the ratio of the dissociation and association rate constants, k_d and k_a, respectively [6]. The rate constants are determined by direct curve fitting of sensorgrams [response units (RU) as a function of time] thanks to dedicated softwares. Noteworthy, an accurate determination of the analyte concentration is crucial for determining the rate and the equilibrium constants of a reaction. A very useful method named Calibration Free Concentration Analysis (CFCA) was developed, which allows the concentration that relates to the specific binding

Abbreviations: B2m, Beta-2 microglobulin; BCA, Bicinchoninic acid assay; CCFCA, Capture Calibration free concentration analysis; CFCA, Calibration free concentration analysis; DSA, Donor specific antibody(ies); HLA, Human leucocyte antigens; mAb(s), Monoclonal antibody(ies); RU, Response units; SCK, Single cycle kinetics; SPR, Surface plasmon resonance

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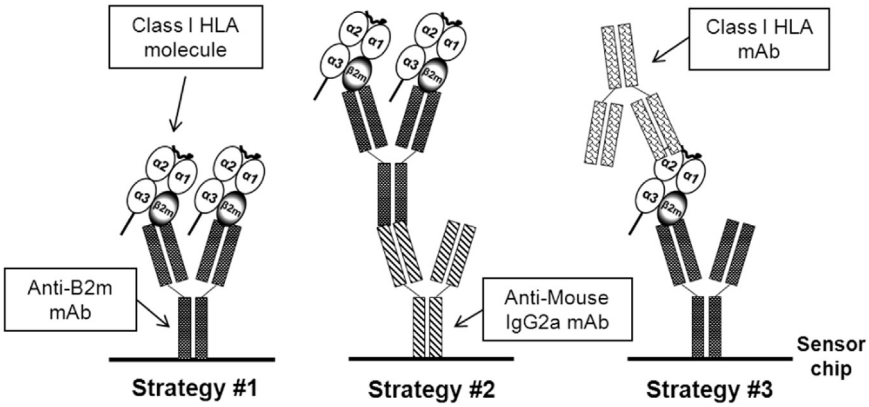

Fig. 1. Strategies used for capture CFCA validation and application to class I HLA antibodies. Strategy #1 corresponds to a covalent CFCA whereas strategies #2 and #3 to capture CFCAs.

activity of an interacting compound to be determined, namely the active concentration [7–10]. Also, a major advantage of CFCA is that a standard curve is dispensable for measuring concentrations, as it only relies on the change of initial binding rates with varying flow rates under conditions of limited mass transport.

Human Leucocyte Antigens (HLA) are cell surface proteins whose main function is to present endogenous and exogenous peptides to T lymphocytes, in order to activate effector and regulatory functions of these immune cells [11]. Class I HLA molecules (HLA-A, -B and -C) are heterotrimers constituted by a polymorphic heavy chain associated to a monomorphic light chain, the beta-2 microglobulin, and a peptide. The exposure to mismatched HLA molecules during pregnancy, after transfusion or upon organ transplantation can lead to the production of allogenic HLA antibodies. These antibodies recognize the amino acids located at polymorphic positions which are different between the subject and the foreign HLA molecules. HLA antibodies are deleterious for organ transplant function and survival when they are donor-directed (the so-called donor-specific HLA antibodies or DSA) [12–15].

To date, the most commonly assay used to determine whether a DSA is present or not is the Single Antigen flow bead assay. It allows patients' sera to be studied with the 200 most frequent class I and class II HLA alleles, with high resolution and sensitivity thanks to a multiplex principle and a fluorescent read out, respectively [16–18]. This assay is most often used in a semi-quantitative fashion, considering that the measured fluorescence value trustfully mirrors the pathogenic strength of the DSA, but this has not been validated yet [19].

Therefore, a real quantitative measurement of HLA antibody concentration and affinity with SPR would be an important step towards deciphering the pathogenic potential of DSA. However, HLA molecules are not covalently linked heterotrimers. Therefore, if covalently immobilized on a sensor chip surface, we can reasonably expect them to be sensitive to the chemicals used for regenerating the functionalized surfaces, like, as a well-known example, low pH solutions [20,21]. Indeed, in our hands, the regeneration solutions that were efficient for dissociating antigen-antibody complexes also irreversibly altered the antibody binding capacity of the HLA target immobilized on the sensor chip surface. In addition, covalent immobilization would drastically reduce the screening capability of the assay which is performed with instruments having only four flow-cells, such as those from Biacore™, given that hundreds of different HLA molecules exist. A capture approach would actually be the most convenient solution to circumvent these impediments, by using a regenerable monoclonal antibody (mAb) to capture HLA molecules through a monomorphic epitope, before injecting the HLA antibody to be characterized.

In this work, using the HLA/HLA antibody system as an experimental model, we demonstrate that the SPR CFCA method can also be used in a capture format (CCFCA for Capture CFCA). CCFCA is of general use and clearly expands the use of SPR for measuring the active concentration of analytes recognizing ligands that cannot be covalently attached to sensor chip surfaces, such as HLA molecules. In addition the capture mode allows determining the kinetic constants of multiple analyte-ligand complexes on the same flow cell, increasing experiments throughput while reducing sensor chips consumption.

2. Material and methods

2.1. Antibodies and HLA molecules

The pan class I anti-HLA W6/32 and anti-beta2-microglobulin (anti-B2m) (clone B2M-01) mouse mAbs, of the IgG2a isotype, were purchased from ThermoFisher Scientific (Rockford, IL). Both mAbs recognize all class I HLA molecules in their native form only. The rat anti-mouse IgG2a (clone R11-89) mAb was purchased from BD Biosciences (Le Pont de Claix, France). The purified HLA-A*02:01, A*11:01 and B*44:02 (One Lambda Inc., Canoga Park, CA) were dialyzed against phosphate buffered saline with 0.05% Tween (PBS-T) (Sigma-Aldrich, St Louis, MI) with a 20 kDa cut-off Float-A-Lyzer G2 device (Spectrum Laboratories, Rancho Dominguez, CA).

2.2. Surface plasmon resonance experiments

SPR experiments were performed at 25 °C with a Biacore™ T200 apparatus (GE Healthcare Life Sciences, Uppsala, Sweden) on CM5 and CM7 sensorchips (Biacore™). The sensorgrams were analyzed with the Biacore T200 Evaluation Software. Antibodies were immobilized by amine coupling using a mixture of N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl carbodiimide according to the manufacturer's instructions (GE Healthcare), after dilution in a 10 mM, pH 5, sodium acetate buffer (10 mM, pH 5), followed by an injection of ethanolamine (1 M, pH 8.5, GE Healthcare) to deactivate the sensor chip surface. A flow cell left blank was used for double-referencing of the sensorgrams [22]. Spikes still present after this step were not removed because they did not affect the results, as previously shown [23,24]. The samples were prepared in PBS-T (Sigma-Aldrich), which constituted the running buffer. Surface regeneration was achieved with a 1 min pulse of 10 mM glycine pH 2.1 (GE Healthcare) followed by a 1 min pulse of running buffer.

2.2.1. Concentration measurements

CFCA experiments were performed with or without