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A versatile method to measure the binding to basic proteins by surface plasmon resonance

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ABSTRACT

Biomolecular interaction is a fundamental mechanism involved in many critical biological processes including gene transcription, translation, and cell signaling networks. Many basic proteins, such as histones, transcription factors, and ribosomal proteins, participate in the interaction of these processes. Surface plasmon resonance (SPR) has been used as a "gold" standard to measure biomolecular interactions. One key issue in SPR assay is how to immobilize ligand without affecting its conformation and biological activity. In this study, we developed a novel method for measuring bindings to basic proteins by SPR, wherein the naturally positive charge of basic protein was utilized to immobilize ligand. The electrostatic interaction between the basic proteins and the negatively charged C1 chip surface (Biacore, GE) generated a specific and stable immobilization without any modification; sodium dodecyl sulfate was identified to be efficient enough for the complete regeneration that allows fresh ligand to be immobilized in each cycle for an optimal kinetic assay. With those parameters determined, an efficient, fast, and reversible method was established to measure bindings to basic proteins under physiological conditions. This new method is widely applicable to the study of binding kinetics between protein–, DNA–, or RNA– and basic protein.

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SPR[1] is an efficient method for measuring biomolecular interactions that are broadly used in basic research and drug development [1]. Compared to other methods studying protein interaction, such as direct protein interaction in vitro and coimmunoprecipitation, SPR is a more sensitive and quantitative biophysical approach that can measure binding affinity and kinetics simultaneously [2]. Real-time and label-free assay is another advantage of the SPR technique. One of the major challenges for SPR assay is how to immobilize ligand without affecting its biological activity [3], which is a common issue for all types of SPR assays. Although SPR is considered as a label-free technique, the immobilization of ligand to sensor chips through different chemical reactions is a kind of "labeling" that could potentially perturb ligand conformation, resulting in nonphysiological interactions with analyte. Currently, amine coupling is the most commonly used method for immobilizing ligand through covalent reactions between the carboxyl group on the sensor chip and the free amine groups from ligand that could reside in any regions of a protein; hence it is more likely to cause certain conformational changes and restrain the free movement of ligand during binding. Other immobilization methods with the Biacore (GE Healthcare) biosensor system are considered better in label-free assay, including indirect capture and affinity tagging (e.g., antibody, His-, GST-, streptavidin-tag) [4–7]. In indirect capture through antibody, if the antigenicity epitope is involved in the interaction with analyte, the immobilization via antibody will compete for the interaction with analyte and decrease the binding. In affinity tagging immobilization, tags added to ligand could potentially alter its protein conformation; the location at N- or C-terminus of a protein also makes a difference in the restriction of ligand orientation. Therefore, those methods are all possible for modifying ligand conformation and activity during immobilization.

Basic proteins are proteins with their p*I* higher than physiological pH; they represent a large group of proteins in the eukaryotic genome that participate in critical biological processes, such as gene transcription and mRNA translation. One dramatic example is that all five core histones (H1, H2A, H2B, H3, and H4) are basic proteins [8]. Most ribosomal proteins are also basic proteins [9]. There are about 2600 DNA binding proteins [10] and over 500 RNA binding proteins [11] in the human genome, among which many of them are also basic proteins. Thus far, there are about 460 basic proteins (p*I* defined from pH 8.0 to 12) that are experimentally identified and can be searched through the ExPaSy database [12]; many of those basic proteins are involved in important physiological processes, such as PAR2 (protease-activated receptor 2) and MBP (myelin basic protein). It is interestingly recognized that most of those basic proteins function via intermolecular

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[1] *Abbreviations used:* AF1, activation function domain 1; GR, glucocorticoid receptor; NTA, nitrilotriacetic acid; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; r-protein, ribosomal protein; TBP, TATA-box binding protein.

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interactions with DNAs, RNAs, or proteins, hence driving us to develop a SPR method that can accurately measure the bindings involving basic proteins.

TATA-box binding protein (TBP), a typical basic protein transcriptional factor, was chosen to develop this method. Immobilization of TBP through amine coupling to CM5 chip and through His tag to NTA (nitrilotriacetic acid) chip failed to measure the binding to GR (glucocorticoid receptor) AF1 (activation function domain 1), a known interaction in GR signaling [13]. The positive charge of TBP at physiological pH was then utilized for the immobilization to the negative charge of the SPR chip surface directly through electrostatic interactions. The C1 chip was found to be optimal in maintaining the physiological conformation of TBP that resulted in a high binding activity to GR AF1. SDS (sodium dodecyl sulfate) was identified to be a simple and efficient regeneration condition, thus establishing a reliable method for measuring bindings to basic proteins. The method was proved by additional experiments with other basic proteins, including histone H2B (basic protein)–5S rDNA and ribosomal proteins (basic protein)–16S rRNA interactions. Hence, this new method is widely applicable for studying macromolecular interactions with basic proteins.

Materials and methods

Materials

TATA-box binding protein and the N-terminal activation function domain of glucocorticoid receptor were prepared as described [14,15]. Histone H2B (*Xenopus*) cDNA subcloned in pET3a was overexpressed in *Escherichia coli* (BL21(DE3)pLysS) and purified by gel filtration and ion-exchange chromatography as described [16]. 5S rDNA (*Lytechinus variegatus*) was prepared as described [17]. 16S rRNA and 30S ribosomal proteins (*E. coli*) free from rRNA were prepared according to the method described by Maguire et al. [18]. All chemicals used in this study were at analytical grade and purchased from Sigma–Aldrich (USA).

SPR binding assay

All SPR experiments were carried out on a Biacore X-100 plus system (GE Healthcare). Basic proteins (e.g., TBP, histone H2B, and ribosomal proteins) were prepared in the binding buffer (10 mM M Hepes, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.05% Tween 20) and directly applied to a Fc2 channel of a CM5 or C1 chip for immobilization. The stability of ligand was tested by a prolonged wash with the binding buffer and other stringent conditions [e.g., 2.5 M NaCl, 1 M $MgCl_2$, and 0.2% NP-40 (4-nonylphenyl-polyethylene glycol)] prior to real binding assay. The ligand was normally immobilized at 200–300 RU to reduce mass transport limitations for reliable kinetics assay. The Fc1 channel was equally treated but without protein as the control. A multicycle kinetics procedure was utilized to measure the binding. Analytes at appropriate ranges of concentrations as indicated in the figures were injected through Fc1 and Fc2 channels to measure the binding. The flow rate was set at 30 μl/min. Data from 120 to 180 s of association and 180 s of dissociation were collected. The sensor surface was regenerated by 0.3% SDS to allow immobilization of fresh ligand for the next cycle of binding. The sensorgram of assay channel (Fc2) was double-subtracted by the buffer control and the Fc1 channel control, and then overlaid for kinetic fitting to obtain the binding on (*ka*) and off (*kd*) rate and affinity ($K_D = kd/ka$). The kinetic fitting was carried out with Biacore X-100 evaluation software using 1:1 Langmuir binding model (A + B = AB) or two-state model (A + B = AB = AB∗) or other models depending on the complexity of binding [19]. χ^2 is used as a statistical measure to evaluate how closely a model fits the experimental data. In general, a χ^2 value lower than 10 is considered as a good fit to sensorgrams with normal noise level. All experiments were repeated 2–4 times, and the average K_D with standard deviation (SD) is shown.

Results

Basic protein can be efficiently immobilized to C1 chip as a ligand

While studying a known protein–protein interaction between TBP and GR AF1 using the SPR method through amine coupling, we observed that pH scouting showed an unusual pattern for immobilization of TBP onto the CM5 chip, and the association of TBP was too strong to be regenerated by basic conditions (e.g., 10 mM NaOH) (Fig. 1A), thereby limiting our ability to continue the measurement with the amine-coupling method. However, it was noted that a large amount of TBP (~7000 RU) was retained on the CM5 chip after pH scouting, suggesting that the positive charge of TBP is sufficient enough to form a stable interaction with the negatively charged CM5 chip surface. The immobilized TBP was actually very stable tolerating the regeneration under stringent conditions, including 2.5 M NaCl, 1 M $MgCl_2$, 50 mM NaOH, and 0.3% SDS (data not shown). Thus, we attempted to use the bound TBP to measure the binding to AF1, a positive result was unexpectedly obtained as shown in Fig. 1B, suggesting that TBP is active via electrostatic immobilization and can bind to AF1 in this SPR assay setting. Furthermore, 0.1% SDS was identified to be effective enough for the complete regeneration, thus maintaining a constant amount of TBP on the chip during the whole binding assay. However, it was noted that the ligand activity was very low at 1.9% (ligand activity = R_{max}/R_L, where R_{max} is maximum binding response, and R_L is the amount of immobilized ligand), suggesting that this electrostatic interaction might be too strongly caused by a pH much lower than the p*I* of basic protein, thus leading to the inactivation of ligand possibly by multiple degrees of interlock between the TBP protein and the negatively charged dextran chains. In addition, such high amount of TBP ligand unexpectedly from pH scouting, instead of using a single pH buffer, cannot be used for kinetic assay. As shown in Fig. 1B, the binding response was not dose dependent; i.e., the response was not proportional to concentration. Two more experiments were repeated in a slightly different way where a single neutral pH buffer was used to immobilize TBP. The reproducible results were obtained and the data were fit with a 1:1 binding model; the affinity (K_D) with SD is shown in Fig. 1B. In sum, this unexpected discovery with positive interaction between TBP and AF1 stimulated us to develop a new binding method based on the strong electrostatic/ionic interaction for ligand immobilization.

Based on the results from Fig. 1, we proposed a general principle for our assay development as illustrated in Fig. 2, where the positively charged basic protein interacts with the negatively charged CM5 or C1 chip surface automatically. A part of ligands is active as shown by its interaction with analyte; others could be inactivated due to the excessive interaction at multiple sites, similar to the immobilization by covalent reactions. Tested first with TBP at p*I* 10.3, we predicted that its net positive charge at neutral pH should be strong enough to interact with the CM5 chip. Now, TBP was dissolved in the binding buffer at pH 7.4 (see Materials and methods) and applied directly to a CM5 chip for immobilization without pH change like that in Fig. 1. The immobilization did occur successfully, and the target amount of ligand reached 200 RU. This low density of ligand was used here and in the rest of the experiments to ensure accurate kinetic assay according to mass transport limitation theory [20]. However, no interaction with AF1 was observed (data not shown), which was correlated