Surface Stabilization Following Indirect Capture of a Binding Partner

Introduction

NPOI technology utilizes heterogeneous binding assays for accurate measurement of binding kinetics. Immobilization of a receptor binding partner is often achieved by direct covalent cross-linking to a SKi Sensor surface. However, in certain situations it is more beneficial to immobilize a protein indirectly via a capture agent. First, it may be necessary when direct immobilization causes a protein to lose its binding capacity. Second, assays may be designed in a way that the protein to be immobilized is captured in a proper orientation for ligand binding. Typical approaches for indirect capture include Biotin/Avidin family proteins, Proteins A,G, A/G/Immunoglobulin, Poly-His/NTA-Ni, and also the use of antibodies against purification tags on recombinant proteins or antibodies that are specific for a certain epitope of the protein to be captured and do not interfere with ligand binding.

A stable binding surface is essential for accurate measurement of binding kinetics. Indirect capture of a receptor binding partner is followed by its dissociation from the capture agent. This dissociation is immaterial in the case of biotinylated molecule captured by Streptavidin or other Avidin-like protein. In other cases dissociation may be slow enough that its influence on the binding kinetics is minor. However, in many instances dissociation of the captured binding partner can not be ignored. For example, some monoclonal antibodies bind loosely to proteins A or G, and many His-tagged proteins dissociate quickly from NTA-Ni surface often with a K_D not better than 1 μM. Also in the case of very high affinity binding pairs a stable binding surface is an absolute requirement. Here we show two examples when indirect capture of a binding partner was combined with chemical cross-linking in order to obtain stable binding surfaces.

Method

Anti-EGF monoclonal antibody was captured on a SKi-Sensor Protein G cartridge in PBS (pH 7.4). For chemical cross-linking antibody capture was followed by injection of 5 mM BS^3 in the same buffer for 5 minutes. Residual active sites were quenched with 1M Ethanolamine (pH 8.5). For His_6-CypA capture, the SKi-Sensor NTA cartridge was charged with Ni^{++} in 0.25M nickel sulfate in 0.3M Tris-HCl for 10 minutes followed by His_6-CypA injection in 250 mM PBS (pH 7.4), 50 μM EDTA, 0.05% P20, and 2% ethanol.

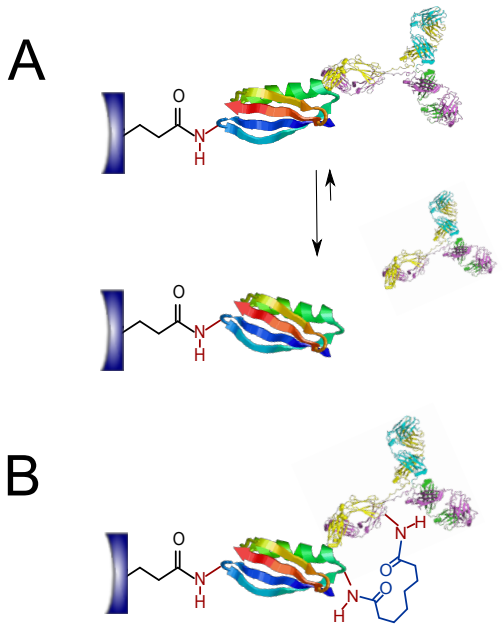

Figure 1: Antibody first captured by Protein G (**A**), not being permanently attached, dissociates in time, especially during subsequent wash steps. Chemical cross-linking with BS^3 after antibody capture results in a stable binding surface with well oriented antibody **B**. The BS^3 reagent is shown in blue.

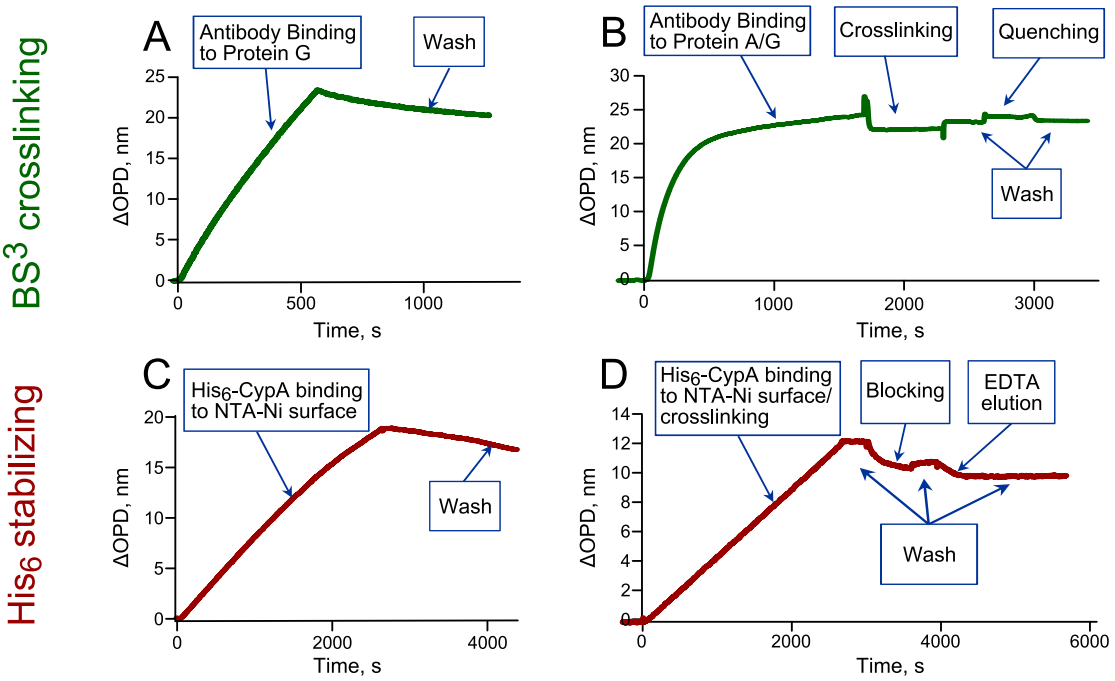

Figure 2: Two separate stabilizations schemes are shown, first for stabilizing a protein-protein interaction BS^3 and a scheme for stabilizing a NTA-Ni interaction through EDC-mediated surface linking. In **A** mouse monoclonal anti-EGF antibody was captured on the Protein G surface; **B**. Antibody capture was followed by chemical cross-linking. **C**. His-tagged Cyclophilin A (His_6-CypA) was captured on the NTA-Ni chip; **D**. His-tagged His_6-CypA was captured on the NTA-Ni chip and simultaneously cross-linked to the surface.

For His_6-CypA capture with simultaneous cross-linking chip surface was activated with 200 mM EDC/50 mM sulfo-NHS in water for 10 minutes following Ni^{++} charging. After His_6-CypA capture residual active sites were quenched with 1 M Ethanolamine (pH 8.5) and unreacted protein was eluted with 0.3 M EDTA (pH 8.0).

Hexahistidine cyclophilin A (His_6-CypA) was a gift from Dr. Martin Wear, University of Edinburgh. Bis[sulfosuccinimidyl] suberate (BS^3) was purchased from Thermo Scientific, Rockford, IL. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO. All experiments were done on SKi-Pro X10 flow cell instrument.

Results

Antibody capture with Protein G

A monoclonal antibody to human Epidermal Growth Factor (EGF) was evaluated as a possible analytical agent. Direct immobilization to carboxyl SKi Sensor surface resulted in a loss of antigen binding capacity (data not shown). Capture on a Protein G-coated chip was followed by dissociation that was too fast for accurate measurements of antigen binding kinetics (Fig. 2A). In order to stabilize antigen-binding surface antibody capture was immediately followed by injection of a cross-linking agent $BS3BS^3$ (Fig. 2B). The remaining BS^3 -derived active sites were quenched and the resulting binding surface was perfectly stable. Immobilized antibody bound human EGF at 65% of theoretical maximum (data not shown) which indicates that chemical cross-linking did not significantly affect complimentarity-determining regions.

His-tagged protein capture on NTA-Ni surface

His-tagged Cyclophilin A (His_6-CypA) had to be immobilized on a chip surface in order to characterize its interaction with Cyclosporine. Direct covalent immobilization on the carboxyl SKiSensor chip resulted in a loss of binding, possibly due to incorrect orientation. Alternatively, His_6-CypA was captured on NTA-Ni chip (Fig. 2), but protein dissociation from the surface was

too fast for subsequent Cyclosporine binding experiments. In order to stabilize NTA-Ni-bound protein the residual carboxyl groups on the chip were activated with EDC/sulfo-NHS mix (Fig. 2). That resulted in simultaneous protein capture in a proper orientation and its covalent cross-linking to the surface. Following that, remaining active sites were blocked and EDTA wash removed the protein that was bound to NTA-Ni but not covalently cross-linked. That procedure resulted in a stable CypA surface (Fig. 2) that retained capacity to bind Cyclosporine (data not shown).

Discussion

Two approaches for immobilization of a binding partner on a chip surface, direct covalent cross-linking and indirect capture, have their own important advantages. While direct immobilization provides a stable binding surface, indirect capture can be designed to present the binding partner in a proper orientation. We demonstrated here that both approaches can be combined in a single run and result in a stable surface best suited for further binding studies.

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