SKi Sensors—4FB biochips

Summary

4FB[1] biochips—available for use with SKi Pro, a fluorescent-free biomolecular interaction analysis platform—are described. These chips make use of a new hydrazone based, conjugation chemistry to reproducibly and covalently generate higher-density protein coverage on their benzaldehyde-functionalized surfaces. Using batch-functionalization of receptor protein with an aromatic hydrazone, it is now possible to reproducibly generate receptor surfaces with minimal day-to-day variance at neutral pH.

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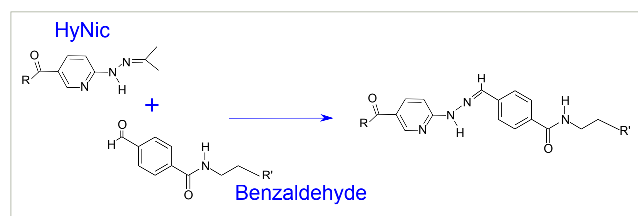
Introduction

Sophisticated kinetic experiments, as well as large-scale, high-throughput protein-interaction analyses require a receptor deposition method that shows little variance between individual depositions. While traditional immobilization depositions, such as EDC/sulfo-NHS[2] chemistries with carboxyl functionalized surfaces, have traditionally been used, they are often plagued by batch-to-batch reproducibility issues due to the instability of activation reagents in aqueous solution. The crosslinker for the 4FB chips, on the other hand, is stable in aqueous solutions over several hours or days, or indefinitely when stored at between -20 and -80°C.

When performing nanoporous optical interferometry (nPOI) based kinetic studies with SKi Pro, a receptor biomolecule is immobilized on a surface. With 4FB chips that is affected in two steps. First the receptor is crosslinked with HyNic[3], an aromatic hydrazone compound. This makes it possible to label large batches of protein with a reagent that functionalizes the protein with an aromatic hydrazone, and to then aliquot and freeze this conjugate. The hydrazine formed from an aromatic hydrazone in aqueous solution will react with a benzaldehyde moiety on the 4FB surface by forming a stable, covalent bis-aryl hydrazone. This reaction has the advantage that it occurs at neutral pH (it is thus compatible with pH-labile proteins) and that it generally leads to higher surface packing densities than other receptor immobilization methods. Given the much lower variance in immobilization efficiency between different experiments, it is therefore suited for applications where large datasets need to be compared across different chips, or where a higher packing density is needed.



In addition to the low variation, the nature of the conjugation of HyNic crosslinked biomolecules to the surface is expected to differ when compared to using carboxyl chemistry. Carboxyl chemistry, using EDC/sulfo-NHS activation, will form a very stable peptide bond with the free amines on a protein. Consider streptavidin with an activated carboxyl chip as shown in Figure 1. Here the conjugation could in principle occur with any of the eight lysines or with the amino terminus of the protein. On the other hand, with a protein crosslinked with a stoichiometric amount of HyNic,

[1] 4-formyl-benzoate
[2] 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/3-sulfo-N-hydroxysuccinimide
[3] 6-hydrazinonicotinate acetone hydrazone

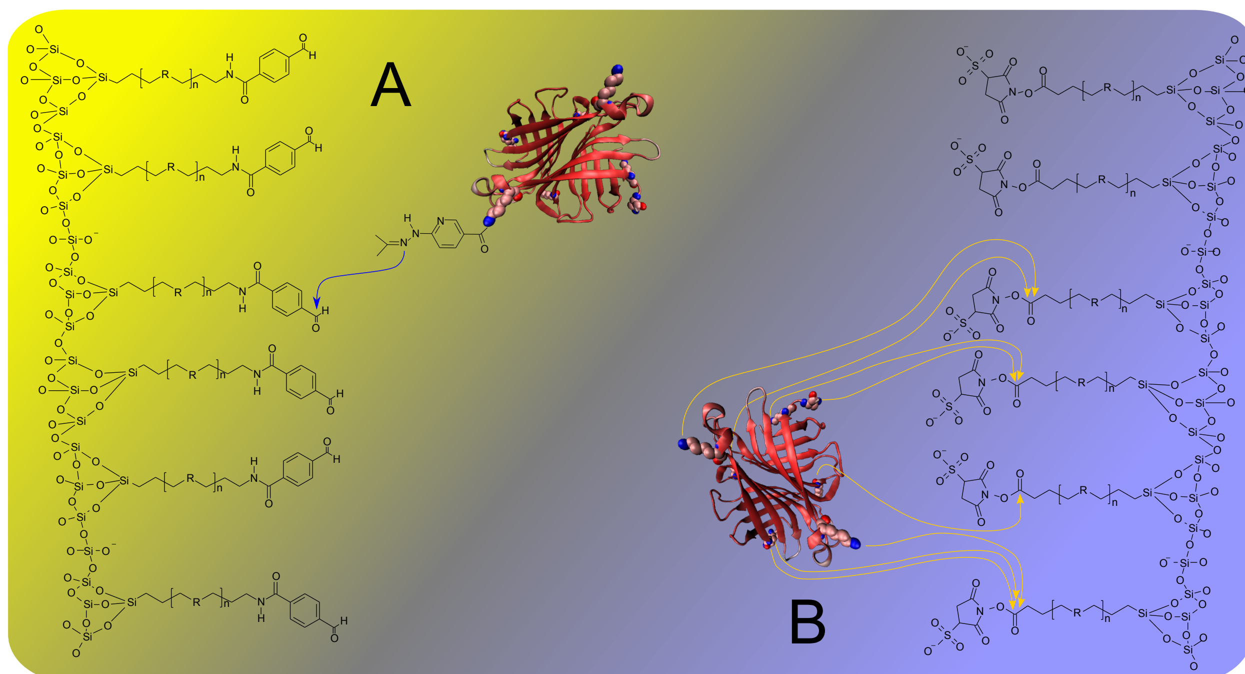


Figure 1: Comparison of hydrazone (**A**) and carboxyl (**B**) immobilization chemistries with core streptavidin—showing highlighted lysine residues (protein data bank ID 1MK5). The HyNic cross-linked protein approaches the 4FB surface and may link to the surface through its hydrazone group. In the case of the activated carboxyl groups, there are multiple options through which the protein may link to the surface.

only the hydrazone moiety would be expected to rapidly react with the 4FB surface.

In the current study we demonstrate the utility of 4FB biochips by functionalizing streptavidin (a biotin interacting protein) with HyNic and reacting the reagent to the 4FB porous silicon biosensors using SKi Pro with the SKi AutoPrep and SKi AutoFlow, flow cell modules. We further test the covalently conjugated streptavidin surface for its ability to bind biotinylated bovine serum albumin (bBSA), a biotinylated DNA hairpin and for any non-specific binding activity.

Method

To produce HyNic functionalized streptavidin, 5 mg of lyophilized streptavidin is dissolved in 0.5 mL of water. The dissolved streptavidin is equilibrated into PBS buffer, pH 7.2, using ZEBA columns. 1 mg S-HyNic[4] is dissolved in 0.03 mL anhydrous DMF. After complete solubilization of the S-HyNic reagent, 15 μL of the solution is added to the dissolved protein, followed by immediate rapid vortexing. After incubation of the labeling reaction at room temperature for 4 hours, unincorporated S-HyNic reagent was removed and buffer exchanged into PBS, pH 6.0, using a ZEBA column. If needed, the concentration of the protein solution was adjusted, and aliquots of the now hydrazone-functionalized protein are frozen and stored at -20°C, or directly processed. 50 μL of a 1 mg/mL HyNic-streptavidin solution in PBS, pH 6.0, is applied to a 4FB porous silicon biochip at a flow rate of 10 μL/min, followed by an exchange into PBS, pH 7.2. 50 μL of a 200 μg/mL biotinylated BSA solution is applied at a flow rate of 10 μL/min, followed by a wash step and application of 50 μL of a 10% v/v rat plasma solution diluted into PBS buffer, pH 7.2 to test for non-specific binding. Biotinylated DNA is immobilized on this streptavidin surface by flowing a 0.1 μg/mL biotinylated DNA solution across the chip in a buffer containing 25 mM HEPES, pH 7.2, 1 M NaCl, and 1 mM $MgCl_2$.

Results

A typical immobilization is depicted in Figure 2 **A**, where the differential flow cell with both sample and reference are used. HyNic-labeled streptavidin effi-

[4] succinimidyl 6-hydrazinonicotinate acetone hydrazone

ciently and permanently reacts with the aldehyde of the 4FB chip surface.

We also establish that such a surface is suitable for capturing a biotinylated DNA hairpins (Figure 2 **B**). Here a covalently stabilized hairpin binds to the HyNic-SA on the 4FB surface causing an OPD change of 14 nm in 500 sec.

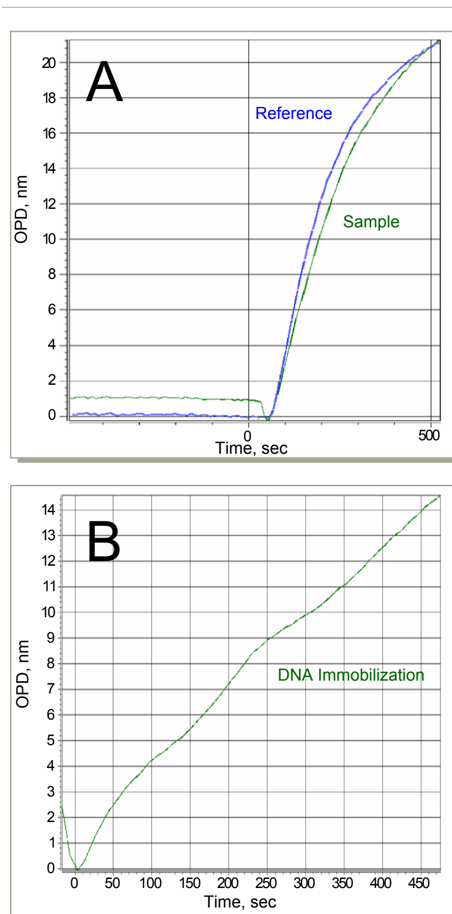


Figure 2: Immobilization of HyNic crosslinked streptavidin upon 4FB biochips using **A** covalent hydrazone chemistry. Those 4FB chips used to capture a DNA hairpin using **B**, the non-covalent biotin/streptavidin interaction.

A typical binding experiment is shown in Figure 3. The stability of the hydrazone bond may be seen in the stable baseline during the post-immobilization washing. A typical experiment results in an OPD shift of 32 nm, which suggests a surface coverage of 40%. The now streptavidin coated surface can efficiently recruit biotinylated BSA from solution, until an equilibrium is reached. The displacement is typically 13 nm. Application of 10% rat plasma does not yield any significant increase in binding activity, suggesting that the 4FB biochip is well suited for applications where elimination of non-specific binding is paramount.

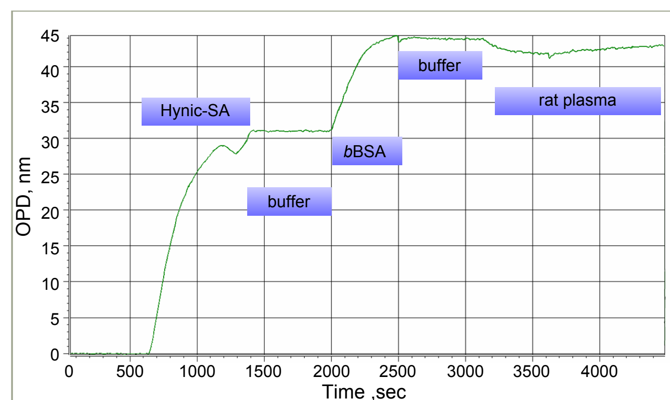


Figure 3: Full immobilization and binding run with non-specific binding test using a 4FB biochip and hydrazone cross linking. The signal shown is the result of subtracting the sample channel from the reference channel from the differential flow cells used with SKi Pro.

Discussion

Using a novel benzaldehyde surface, together with hydrazone-functionalized protein, it is now possible to efficiently and reproducibly generate receptor surfaces for kinetic and high-throughput applications, where minimizing sample-to sample, or chip-to-chip variability is critical. This approach also allows one to carefully establish protein functionalization conditions coupled to activity based enrichment methods (e.g. affinity purification of antibodies) to ensure that the immobilized receptor retains biological function, prior to immobilization on the porous silicon surface. Furthermore, we demonstrated that we can immobilize biotinylated DNA molecules to a streptavidin surface, thus opening up the SKi Pro instrument platform to study protein-DNA interactions.

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