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# Self-Assembled N-Heterocyclic Carbene-Based Carboxymethylated Dextran Monolayers on Gold as a Tunable Platform for Designing Affinity Capture Biosensor Surfaces

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**ABSTRACT**

Sensor surfaces play a predominant role in the development of optical biosensor technologies for the analysis of biomolecular interactions. Thiol-based self-assembled monolayers (SAMs) on gold have been widely used as linker layers for sensor surfaces. However, the degradation of the thiol-gold bond can limit the performance and durability of such surfaces, directly impacting their performance and cost-effectiveness. To this end, a new family of materials based on N-heterocyclic carbenes (NHCs) has emerged as an alternative for surface modification, capable of self-assembling onto a gold surface with higher affinity and superior stability as compared to the thiol-based systems. Here we demonstrate three applications of NHC SAMs supporting a dextran

layer as a tunable platform for developing various affinity capture biosensor surfaces. We describe the development and testing of NHC-based dextran biosensor surfaces modified with each of streptavidin, nitrilotriacetic acid, and recombinant Protein A. These affinity capture sensor surfaces enable oriented binding of ligands for optimal performance in biomolecular assays. Together, the intrinsic high stability and flexible design of the NHC biosensing platforms show great promise and open up exciting possibilities for future biosensing applications.

**Keywords:** N-heterocyclic carbenes, NHCs, surface plasmon resonance, biosensing, carboxymethylated dextran, streptavidin, nitrilotriacetic acid, protein A

## 1. INTRODUCTION

N-heterocyclic carbenes (NHCs) are an important and unique class of neutral, L-type ligands, which have emerged as versatile ligands in organometallic chemistry.[1,2] Their ease of synthesis and ability to form strong bonds with various metals provide an alternative route for the development of functionalized materials.[3–8] Significant recent advances in the development of NHC surface chemistry on planar gold surfaces have been made, due to the higher affinity and stability of NHC self-assembled monolayers (SAMs) on gold as compared to traditional thiol-based systems.[3–8] The NHC-based SAM is resistant to oxidation under conditions which would destroy a thiol-based SAM, meaning that devices incorporating the NHC technology should have longer lifetimes and are usable under a wider range of environments.[1–4] These advances have increased the practicality and desirability of developing NHC-supported biosensor surfaces for use in biosensing applications.[9,10]

Surface plasmon resonance (SPR)-based biosensing technology has been widely used to provide detailed insights regarding the affinity and kinetics of biomolecular interactions.[11–16]

Compared to other technologies, SPR offers significant advantages, including real-time monitoring, label-free detection, reusable sensor surfaces, flexible assay design, accurate sample handling, and minimal sample consumption.[11,16] With the ligand immobilized on the sensor surface, the analyte is made to flow over the ligand surface and the binding event can then be monitored and plotted as a function of time.[12,13] A critical component of the SPR biosensor is the sensor surface which is used to immobilize a ligand of interest.[11–15] Molecular architectures with well-defined functionalities and compositions on sensor surfaces allow for an increased range of biosensing applications.[13,15] Thus, the robust attachment of NHCs on gold offers the potential for further advances in chemical modifications of the surfaces, enabling an expanded range of biomolecular attachment.[4,5,9,10]

Previously, we have introduced the use of NHC linkages to act as a support for constructing biosensor surfaces.[5,9,10] In addition to showing the strong performance of an NHC-based hydrophobic association (HPA) biosensor surface, we demonstrated the feasibility of an NHC-linked carboxymethylated dextran (NHC-CM) surface for biosensor applications. Although the CM surface offers a very versatile platform for the covalent immobilization of many ligands to the biosensor surface, some coupling methods can produce a functional surface with randomly-oriented ligand molecules, which may result in a reduced exposure of the effective binding sites.[13,15] Alternative methods that offer immobilization with homogeneous ligand orientation can improve the activity of the biosensor surface and are therefore desirable. Moreover, in certain circumstances, some biomolecules can be difficult to immobilize by covalent coupling.[13,15,17] For example, negatively-charged ligands such as oligonucleotides undergo electrostatic repulsion to the negatively-charged carboxymethylated dextran surface.[12]

Affinity capture has been shown to be an effective approach to resolve issues with the covalent coupling of ligand molecules in SPR-based biosensing.[13] In affinity capture assays, functional capture molecules are covalently immobilized on the sensor surface.[13,17] This generates a uniform structural orientation of the ligand molecules for subsequent analyte binding assays.[13,14,15,17] The NHC-CM surface offers a highly stable and tunable platform which shows promise for developing various types of these high-affinity coupling methodologies.[5,9,10] The commercial streptavidin chip (SA chip, from GE Healthcare) was developed to take advantage of the high-affinity streptavidin-biotin interaction, whereby biotinylated ligands can be captured onto a pre-immobilized streptavidin surface in a homogeneous orientation through a specific binding site on the streptavidin protein.[13] In addition to the SA chip, there are several other commercially available SPR sensor chips that utilize affinity capture for biomolecular detection. The commercial nitrilotriacetic acid (NTA) chip (GE Healthcare) has a dextran matrix containing pre-immobilized NTA on a gold surface.[13,15] Following injection of a nickel solution, the NTA-modified surface can be used to capture histidine (His)-tagged biomolecules in a largely uniform orientation.[18–20] EDTA can be used to disrupt the binding between the His-tagged protein and Ni-NTA complexes, allowing the repeated use of the sensor surface, effectively eliminating the problem of ligand degradation during the repeated surface regeneration cycles.[18–20] Another example is the Protein A chip (again, pioneered by GE Healthcare) which utilizes a pre-immobilized protein A on the dextran surface to bind antibodies through the heavy chain of their Fc-region, ensuring a specific orientation of the antibodies for subsequent binding assays.[21,22]

The focus of this paper is to explore in depth the functional range of the NHC-based chips, and show that they have at least the same range of selectivity and ease of operation in biomolecular assays as the available commercial platform. In doing so, we report proof-of-concept studies of

the development and testing of three examples of NHC-based affinity-capture biosensor surfaces: NHC-SA, NHC-NTA, NHC-Protein A. The use of NHC ligand as linker for developing affinity capture surfaces has not been done for any such biosensing systems, and thus, we posit that our work is unique and distinct from previous researches based on other traditional organic ligands, such as thiol-based and organosilane-based biosensing systems. Each of these biosensor surfaces is comprised of a 50 nm gold layer on glass, an NHC-linker, a dextran layer, and the corresponding functional modification. In our previous work, we briefly described an NHC-based dextran-linked streptavidin (NHC-SA) surface, which showed nearly identical effectiveness for sensing biotin as compared to the corresponding commercial thiol-coupled streptavidin surface.[5] Here, we extend this concept to a wider scope of applications, including aptamer selection and DNA-protein binding. The NHC-based dextran matrix pre-immobilized with NTA chip (NHC-NTA chip) is developed and employed for its efficacy in binding His-tagged proteins. Finally, the NHC-Protein A chip was developed by pre-immobilizing protein A on dextran-modified NHC surface and tested for antibody binding. Our results demonstrate that NHC-linked surfaces are a robust and versatile platform for evaluating a wide range of biomolecular interactions through SPR-based biosensing.

## 2. EXPERIMENTAL SECTION

### 2.1 Streptavidin-Based Chips

#### 2.1.1 Streptavidin-Aptamer Recognition on NHC-SA Chip

The NHC ligand was prepared according to a previously reported method.[5] The NHC-SA surface was prepared in a Biacore 3000 using the amine coupling method in PBS buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at a flow rate of 5 μl/min. The detailed

preparation procedure of the NHC-CM surface is described in the Supporting Information. Initially, the carboxyl groups on the 6 kDa NHC-CM surface were converted to active esters by a 10 min exposure to a mixture of 1:1 (v/v) 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.05 M hydroxysuccinimide (NHS). Streptavidin was then immobilized onto the activated surface by a 10 min injection of 0.1 mg/ml streptavidin in 10 mM sodium acetate (pH 4.7). The immobilization procedure was terminated by a 10 min injection of 1 M ethanolamine (pH 8.5) to block remaining NHS-esters. This was followed by a 1 min injection of 2 M NaCl at 20 µl/min to remove any unbound streptavidin, resulting in a final streptavidin immobilization level of approximately 2020 response units (RU).

Recognition of streptavidin-specific aptamer (aptamer 29 (7.7 kDa))[23] by the NHC-SA chip surface was compared versus binding to a library of random DNA oligonucleotides (25 kDa),[23] non-SA-specific aptamers (p53R175H-APT-1 (10.8 kDa)[24] and p53R175H-APT-8 (7.7 kDa),[24] avidin (66 kDa), and BSA (66 kDa), which had been previously diluted into the PBS buffer to a final concentration of 50 nM. The aptamer sequences used in the present study are shown in Supporting Information (Table S1). To equilibrate the NHC-SA chip surface, three buffer injections were initially performed at a flow rate of 10 µl/min. The samples were then injected over the NHC-SA surface at a flow rate of 10 µl/min for 3 min, followed by a 2 min dissociation phase. The sensor surface was then regenerated by two injections of 10 mM NaOH for 30 seconds, followed by a 3 min waiting period to monitor baseline stability.

### 2.1.2 DNA-Protein Interactions on SA and NHC-SA Chips

Surface preparation and evaluation experiments were performed using HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % P20, pH 7.4) as the running buffer. Two flow cells

on each sensor chip were used as the active surface and reference surface, respectively. Each active surface was prepared by injecting a solution containing 1:1 (v/v) 0.2 M EDC and 0.05 M NHS for 10 min at 5 µl/min. Streptavidin at 0.1 mg/ml in 10 mM sodium acetate (pH 4.7) was then injected for 10 min and the surface was blocked by a 10 min injection of 1 M ethanolamine (pH 8.5). The reference surface was activated by EDC/NHS and blocked with ethanolamine. Three 2 min injections of buffer were used to remove unbound streptavidin and stabilize the surface. The resultant immobilization levels of streptavidin on the CM3 and NHC-CM chips were approximately 3000 RU and 2000 RU, respectively.

Binding tests were performed at a flow rate of 10 µl/min. A 2.5 ng/ml solution of a biotinylated DNA fragment containing the HetR recognition sequence (from Anabaena sp. Strain PCC 7120)[25,26] was injected over the working and reference flow cells of both the commercial SA and our NHC-SA surfaces, giving rise to immobilization levels of 690 RU and 310 RU, respectively. Three 2 min buffer injections were used to equilibrate the chip surfaces. To evaluate binding to the DNA fragment surface, His-tagged transcription factor HetR, which had been overexpressed in *Escherichia coli* strain Rosetta (DE3),[26] was injected over the reference and biotinylated DNA surfaces in concentrations ranging from 6 to 100 µg/ml. Injections consisted of a 3 min association phase, followed by a 5 min dissociation phase. Regeneration of the SA and NHC-SA surfaces was effected by a 1 min injection of 2 M NaCl. Reference-subtracted data were processed following subtraction of buffer injection sensorgrams (double referencing method).[27]

### 2.2 NHC-NTA Based Chip

#### 2.2.1 Preparation of the NHC-NTA Chip

$N_\alpha$,$N_\alpha$-Bis(carboxymethyl)-L-lysine hydrate was immobilized to the surface of an 500 kDa NHC-CM chip using amine coupling in PBS buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4). The sensor surface was initially activated by two consecutive 10 min injections of 1:1 (v/v) 0.2 M EDC/ 0.05 M NHS at a flow rate of 5 µl/min, followed by a 30 min injection of 10 mM NTA in PBS buffer. The surface was then blocked with a 10 min injection of 1 M ethanolamine (pH 8.5). The resultant NTA immobilization level was approximately 140 RU per flow cell.

### 2.2.2 Validation of NHC-NTA Chip with His-Tagged Proteins

To evaluate the ability of the NHC-NTA sensor surface to capture 6×His-tagged proteins, solutions of proteins were passed over two NTA surfaces: one activated with 0.5 mM $NiSO_4$ (in MiliQ $H_2O$), and a reference where the NTA groups were not exposed to $NiSO_4$. The binding of two 6×His-tagged proteins was tested: 25 µg/ml His-tagged HetR overexpressed in *Escherichia coli* strain Rosetta (DE3)[26] and 50 µg/ml His-tagged Cu/Zn superoxide dismutase (SOD)[28] expressed in *Escherichia coli* strain *BL21* (DE3). For comparison, a non-tagged Cu/Zn SOD was also tested at 50 µg/ml. Surface activation with nickel was performed using a 2 min injection at a flow rate of 10 µl/min. Binding tests for the 6×His-tagged proteins were carried out at a flow rate of 10 µl/min. Injections of 6×His-HetR had a duration of 1 min, followed by a 4 min dissociate phase, whereas injections of 6×His-tagged Cu/Zn SOD had a 2 min association phase followed by a 6 min dissociation. For the non-tagged Cu/Zn SOD protein, injections consisted of a 2 min association and 4 min dissociation phases. Regeneration of the sensor surfaces was effected as follows: one 1 min injection of 350 mM EDTA for His-tagged HetR protein, and 1 min injection of 350 mM EDTA mixed with 200 mM imidazole for His-tagged Cu/Zn SOD and non-tagged Cu/Zn SOD proteins.

## 2.3 NHC-Protein A Based Chip

### 2.3.1 Preparation of the NHC-Protein A Chip

Protein A was immobilized on the 500 kDa NHC-CM sensor surface by amine coupling chemistry in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005 % P20, pH 7.4). The surface was first activated with a 1:1 mixture of 0.2 M EDC/0.05 M NHS for 7 min at a flow rate of 10 µl/min, followed by a 10 min injection of 25 µg/ml protein A in 10 mM sodium acetate (pH 4.4). Finally, the surface was blocked with a 7 min injection of 1 M ethanolamine (pH 8.5). The reference surface was activated by EDC/NHS and blocked with ethanolamine. The resultant immobilization level of protein A was approximately 980 RU. Three 2-min injections of the HBS-EP buffer were used to equilibrate the active and reference surfaces immediately prior to kinetic tests.

### 2.3.2 Functional Tests of NHC-Protein A Chip Using Antibody Binding

Serial twofold dilutions of normal rabbit Immunoglobulin G (IgG) (2.5 to 20 µg/ml) were passed over the active and reference surfaces at a flow rate of 5 µl/min, with association and dissociation times of 3 min and 7 min respectively. The surface was regenerated by a 1 min injection of 10 mM glycine-HCl (pH 1.5) to disrupt the interaction between protein A and rabbit IgG. Reference-subtracted data were processed following subtraction of buffer injection sensorgrams (double referencing method).[27]

Serial twofold dilutions of normal mouse IgG (0.5 to 16 µg/ml) were injected over the active and reference surfaces to assess the binding performance of an NHC-Protein A chip, with a 560 RU protein A immobilization level. Triplicate injections of each normal mouse IgG concentration were performed over the reference and protein A surfaces at a flow rate of 5

μl/min. Each cycle consisted of a 1 min waiting period for monitoring of the baseline stability, then the mouse IgG antibody/protein A complex was allowed to associate and dissociate for 1 min and 3 min, respectively, followed by a 1 min buffer injection to prevent carryover between analyte injections. Between binding injections, the surfaces were regenerated with a 1 min pulse of 10 mM glycine-HCl (pH 1.5). Reference-subtracted data were processed following subtraction of buffer injection sensorgrams (double referencing method).[27]

### 2.3.3 Stability of the NHC-Protein A Chip

A total of 90 binding-regeneration cycles were carried out by injection of normal mouse IgG (2 μg/ml) over the active and reference surfaces of the NHC-Protein A chip at a flow rate of 5 μl/min., with association and dissociation times of 1 min and 2 min respectively. Regeneration was achieved by a 1 min injection of 10 mM glycine-HCl (pH 1.5).

## 3. RESULTS AND DISCUSSION

### 3.1 Validation of NHC-SA Chip

In our previous work, a dextran layer functionalized with carboxyl groups was constructed onto the NHC SAM surface (NHC-CM surface) for covalent coupling biomolecules for general use in biosensing.[5,10] Streptavidin is a tetrameric protein containing four identical binding sites for biotin with a high affinity.[15] We have utilized this specific interaction and developed an NHC-based dextran-linked streptavidin (NHC-SA) surface that was shown to bind biotin effectively,[5] making it possible to detect proteins and other biological analytes in physiological solutions.

Control experiments were first performed to confirm that the observed binding events were due to the specific binding of biotin to streptavidin (Figure S1, in the Supporting Information). First, the addition of a 1 mM D-biotin solution to an unmodified NHC-dextran surface produced a limited change in response (16 RU). Second, the streptavidin-functionalized NHC-dextran surface exhibited a significant increase in response (235 RU) upon addition of same concentration of D-biotin solution. This binding response was stable following the addition of buffer solution, which corresponds to the very small dissociation rate of the streptavidin-biotin system, implying a highly specific binding event. Third, the addition of the same concentration of D-biotin solution to a biotin-saturated NHC-SA surface did not show an appreciable response change (13 RU) relative to the fresh NHC-SA surface. These controls suggest that there is minimal non-specific binding of the D-biotin binding to either bare or streptavidin-modified NHC-dextran surfaces. Thus, the dextran-modified NHC surface can provide a robust means for discriminating against false-positive binding response due to the minimal non-specific binding.

In the following studies, two further validation tests were carried out on the NHC-SA chip, including recognition of an SA-specific aptamer and binding of biotinylated DNA, followed by interaction with a DNA-binding protein.

### 3.1.1 Recognition of Streptavidin-Specific Aptamer on NHC-SA Chip

Aptamers can be used as recognition elements to pre-select targets with high affinity and specificity; therefore, they can provide alternative solutions in biomedical diagnostics, environmental monitoring, and other areas than traditional approaches.[29–31] The development of sensor surfaces with high sensitivity and selectivity to specifically distinguish one type of analyte from another is particularly attractive for use in biological applications.[11]

In the present experiment, the NHC-CM sensor surface was first modified with streptavidin using amine coupling chemistry and used as a binding receptor, then solutions with different samples, running buffer and regeneration solution were alternately delivered to the NHC-SA surface (Figure 1). Recognition of the SA-specific aptamer (aptamer 29) by the NHC-SA surface was compared to the binding levels for a library of random DNA oligonucleotides, non-SA-specific aptamers (p53R175H-APT-1 and p53R175H-APT-8), avidin, and BSA (Figure 1). The aptamer sequences used in the present study are listed in Table S1 in the Supporting Information.

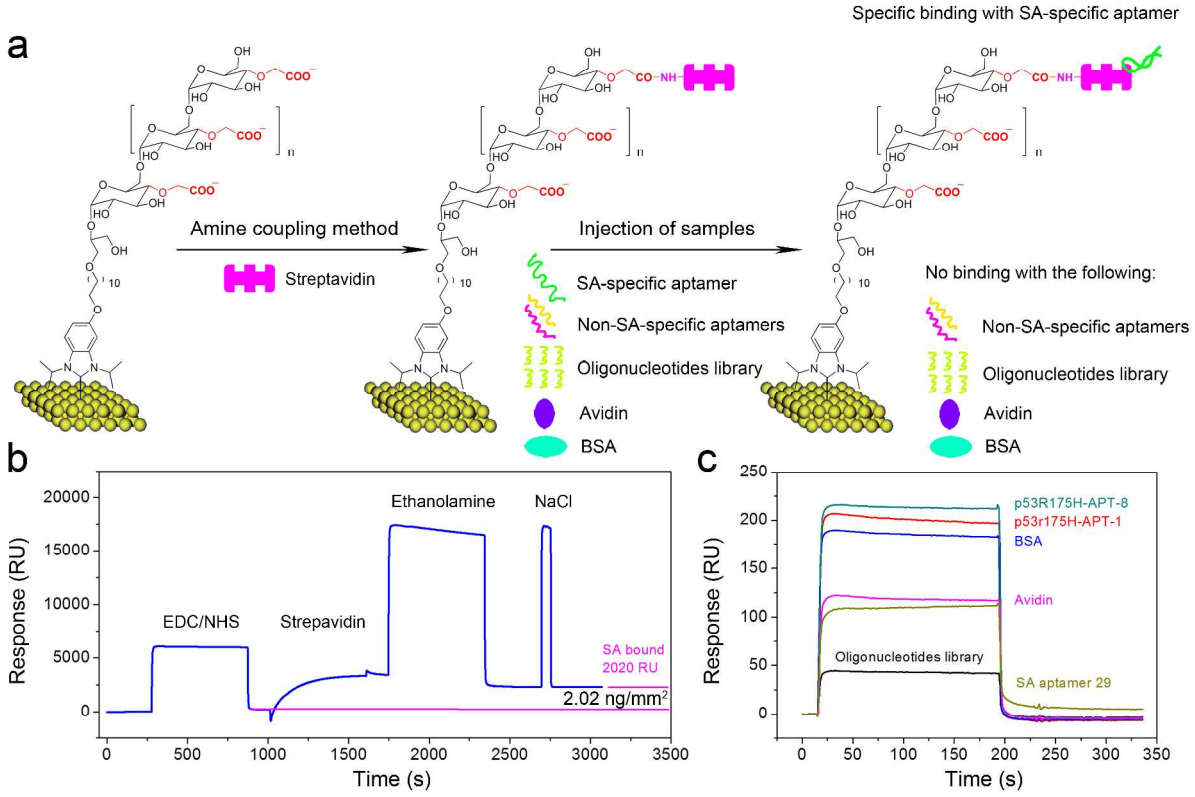


**Figure 1.** (a) Schematic illustration of the preparation procedure for NHC-SA chip followed by recognition of a streptavidin-specific aptamer. Sensorgrams for (b) immobilization of streptavidin (0.1 mg/ml, in 10 mM sodium

acetate, pH 4.7) on 6 kDa NHC-CM chip using amine coupling chemistry, and (c) recognition of streptavidin-specific aptamer on the NHC-SA chip.

Of the analytes tested for propensity to bind to the NHC-SA surface, the most significant binding was observed for the streptavidin-specific aptamer (18.4 RU), and to a lesser extent for the random oligonucleotide library (1.3 RU), as shown in Table S2. Since this oligonucleotide library sample contained a mixture of oligonucleotides from which SA-specific aptamers were selected, the approximately 10-fold lower binding response observed is reasonable (as compared to the pure SA-specific aptamer 29). Furthermore, the level (18.4 RU) of bound SA-specific aptamer (aptamer 29) was proportional to the level (230 RU) observed for an SA-modified CM5 chip in a previous study, when corrected for the analyte injection concentration (50 nM vs 0.5 μM).[23] These observations suggest the highly specific interaction between the SA-specific aptamer and NHC-SA surface, which is consistent with the reported value.[23] By contrast, the comparatively low binding which we observed for the non-SA specific samples supports the use of the NHC surface for screening applications, such as aptamer selection.[32,33] The above results reveal the robustness and potential of the NHC-SA surface to be functionalized with bio-recognition moieties to generate high selectivity to their specific targets.

### 3.1.2 Interaction Test for Biotinylated DNA with Protein Using SA and NHC-SA Chips

Specific protein-DNA interactions play a key role in gene expression and other biological areas.[34] The streptavidin sensor surface has routinely been used to study the binding of nucleic acid-related interactions, employing nucleic acids derivatized with biotin.[35] In the present study, the performance of our streptavidin-modified NHC chip was tested versus a streptavidin-derivatized commercial CM3 chip using the interaction between the protein HetR and biotinylated HetR-specific DNA (Figure 2).

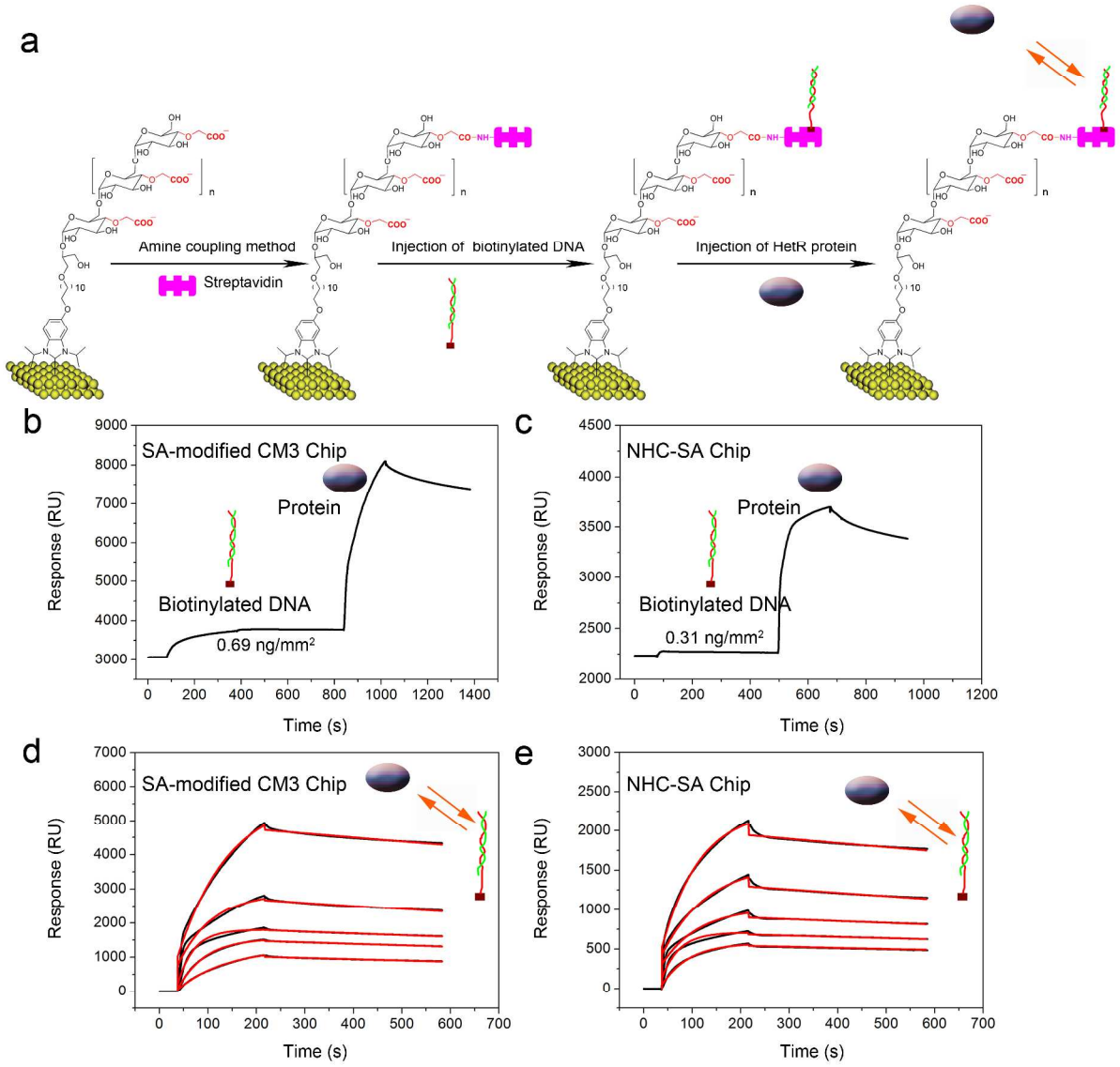


**Figure 2.** (a) Schematic illustration of the preparation procedure for NHC-SA chip, followed by immobilization of biotinylated HetR-specific DNA to interact with transcription factor HetR protein. Sensorgrams showing the immobilization of biotinylated DNA followed by interaction with the HetR protein, using streptavidin-modified (b) commercial CM3-SA or (c) our NHC-SA chips. Reference-subtracted sensorgrams (black lines) and fitting (red

lines) of the HetR protein injections ranging from 6 to 100 µg/ml onto surface pre-immobilized with biotinylated DNA using (d) commercial CM3-SA or (e) our NHC-SA chips.

Figure 2a illustrates the preparation process for NHC-SA chip, followed by immobilization of biotinylated HetR-specific DNA to interact with transcription factor HetR protein.. In the first stage, streptavidin was attached to the chip surface, using the amine coupling described in the previous section. Next, biotinylated DNA was injected and captured onto the streptavidin surface, followed by interaction with HetR protein. Figures 2b and c show sensorgrams corresponding to this second stage. At t = 0 s, buffer solution was introduced over the streptavidin-modified CM3 or NHC-SA surfaces. At t = 80 s, a solution of biotinylated DNA specific to HetR adsorption was injected for 5 min over each sensor surface. The resulting increase in RU in the sensorgram confirms the formation of a streptavidin-biotinylated DNA complex on each sensor surface. The resultant sensor surfaces were then used for studying the interaction between the protein HetR and HetR-specific DNA. Figures 2d and e show the reference-subtracted sensorgrams (black lines) and fitting (red lines) of the process for the target analyte, HetR, interacted with biotinylated DNA.. Solutions of different protein HetR concentrations were delivered to the two pre-immobilized HetR-specific DNA surfaces (at t = 35 s). This was followed by the formation of DNA-protein complex on the sensor surface, leading to an association phase and thus the corresponding association rate constant ($k_a$) can be obtained. At t = 215 s, the protein solution was replaced by the buffer, and the complex was allowed to dissociate, yielding a dissociation rate constant ($k_d$) for the interaction, which was followed by a regeneration step (not shown here).

Although we observed higher binding levels for biotinylated DNA on the SA-modified CM3 chip as compared to the NHC-SA surface (Figures 2b and c), the binding levels and sensorgram shapes for the subsequent binding of the HetR protein were virtually identical for both surfaces

after correcting for the difference in initial DNA binding. The binding data were then fitted using the 1:1 Langmuir binding model between analyte A (HetR protein) and ligand B (HetR-specific DNA).

$$A + B \leftrightharpoons \mathrm{AB} \quad (1)$$

where A represents the solution phase HetR protein, B represents the free surface binding sites, and AB the surface-bound complex. The binding process was assumed to be pseudo-first order without interactions between individual ligand molecules. The net rate of complex (AB) formation during injection is:

$$\frac{d[AB]}{dt} = k_a[A]_t[B]_t - k_d[AB]_t \quad (2)$$

where $k_a$ and $k_d$ are the association and dissociation rate constants associated with Reaction (1).

During the association phase, solutions of an analyte flowed over the ligand surface, and the kinetics of the binding events can be derived from the resultant binding curves. The quantity of HetR-specific DNA bound at time t, $R_t$, is

$$R_t = \frac{R_{max}[A]}{K_D+[A]} \cdot \left(1 - \frac{1}{e^{(k_a[A]+k_d)t}}\right) \quad (3)$$

Similarly, during the dissociation phase (washing the ligand surface with the running buffer alone, [A] = 0), the HetR protein dissociated from the complex, which follows simple exponential decay kinetics:

$$R_t = R_0 e^{-k_d t} \quad (4)$$

where $R_{max}$ is the maximum HetR protein binding capacity, $R_0$ binding response at the start of the dissociation phase, and $K_D = k_d/k_a$ is the equilibrium dissociation constant.

Of note is that Figures 2 d and e demonstrate the high quality of the binding results obtained on both surfaces. In each case, the response intensities are proportional to the injected HetR protein concentrations, along with an exponential trend for the binding curves. Moreover, the responses and the fitting curves are superimposed over each data set, revealing the high reliability of the reported kinetics for the interactions. Kinetic parameters for the HetR/DNA interaction derived from a fit of these data sets also showed a strong similarity between NHC results and those that can be obtained with a thiol-based SA-derivatized CM3 surface and the NHC-SA surface. Specifically, $k_a$ and $k_d$ of the HetR/DNA interaction on SA-modified CM3 chip were determined to be $(9.2 \pm 2.8) \times 10^2$ $M^{-1}s^{-1}$ and $(3.1 \pm 0.4) \times 10^{-4}$ $s^{-1}$, respectively, yielding an equilibrium dissociation constant ($K_D$) of $(3.4 \pm 2.1) \times 10^{-7}$ M. For the interaction on the NHC-SA chip, $k_a$ and $k_d$ were determined to be $(8.6 \pm 1.7) \times 10^2$ $M^{-1}s^{-1}$ and $(2.6 \pm 0.4) \times 10^{-4}$ $s^{-1}$, respectively, resulting in a $K_D$ of $(3.0 \pm 1.1) \times 10^{-7}$ M. These values are comparable to corresponding $K_D$ values for the interaction of pentapeptide RGSGR (PatS5) with HetR.[36] This species prevents the binding of HetR to DNA by a specific HetR-PatS5 interaction with a $K_D$ of $2.27 \times 10^{-7}$ M.[36] Moreover, HetR is known to have much higher affinity for binding with hexapeptide ERGSGR (PatS6) with a $K_D$ of $7 \times 10^{-9}$ M.[37] By comparing these data, the $K_D$ values of the HetR/DNA interaction obtained on the SA and NHC-SA surfaces are consistent with each other and in good agreement with the previous studies, further demonstrating the reliable performance of the NHC-SA surface.

We also investigated the reproducibility of immobilizing streptavidin onto NHC-dextran surfaces, which were used for biotin,[5] SA-specific aptamer, and biotinylated DNA-protein studies, by comparing the corresponding streptavidin immobilization levels. Notably, the binding levels of streptavidin on these surfaces exhibit good consistency, being 2215 RU,[5] 2020 RU, and 2000 RU, respectively, yielding a minimal 5.7 % variation among these NHC-dextran surfaces. The chip-to-chip variation might stem from a small variation of dextran density and carboxylation during our modification process. This could be reduced through further optimization of the modification steps, such as minimizing the difference of the activity of EDC/NHS during the activation process, or through the number of exposed active sites of streptavidin protein. Overall, though, these results strongly support the robustness of uniform NHC-based dextran surfaces for SPR-based biosensing.

### 3.2 Validation of NHC-NTA Chip

The merit of the metal-chelate capture approach is the simple and sterically orientated ligand binding for optimal site exposure for use in biosensing.[12,15]His-tagged proteins can be effectively immobilized by NTA-Ni complexes previously bound to a sensor surface. We, therefore, chose this concept to further benchmark the biosensing ability of an NHC-based SPR sensor chip.

To validate the NHC-NTA chip as an effective immobilization platform for studying biomolecular interactions involving His-tagged proteins of interest, we tested the immobilization of two types of His-tagged proteins and compared them to a non-tagged protein on an NHC-NTA surface (Figure 3).

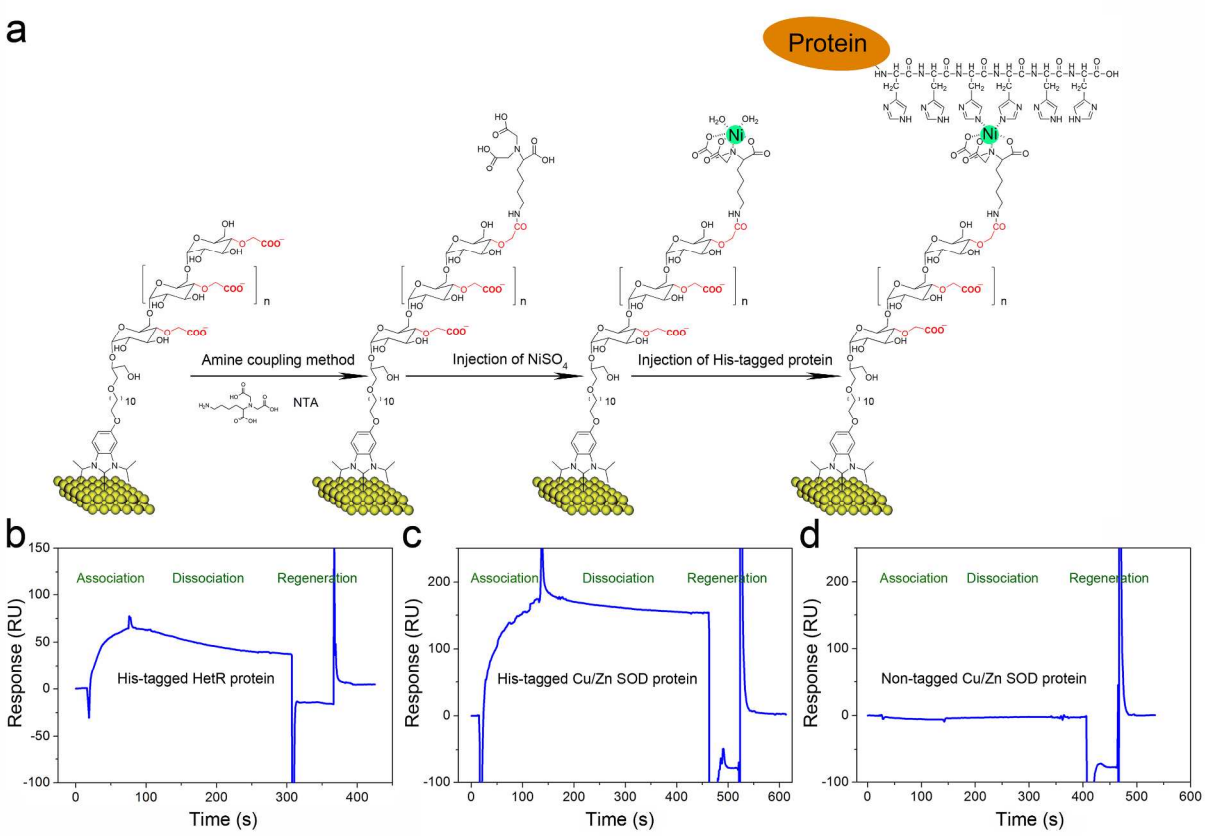


**Figure 3.** (a) Preparation of an NHC-NTA surface from an NHC-supported dextran matrix, followed by surface activation through the formation of NTA-Ni complexes. Reference-subtracted sensorgrams of protein binding to activated NHC-NTA surface: (b) 6×His-tagged HetR, (c) 6×His-tagged Cu/Zn SOD, and (d) non-tagged Cu/Zn SOD.

Figure 3a illustrates the initial process of producing an NHC-NTA surface from our NHC-CM chip via the immobilization of NTA molecules, and the subsequent activation of this surface for the binding of His-tagged proteins by $NiSO_4$ exposure. To evaluate the ability of the NHC-NTA surface to selectively capture His-tagged proteins, two 6×His-tagged proteins (HetR and Cu/Zn SOD) and a non-tagged protein (Cu/Zn SOD) were exposed to two NTA surfaces: one activated with $NiSO_4$, and a non-activated reference surface. The resulting immobilization curves (Figures 3b and c) show that the 6×His-tagged proteins both showed affinity for the $Ni^{2+}$-activated NTA surface and that their immobilization levels approached equilibrium levels within 5 min of the protein injection. The corresponding $K_D$ values obtained by fitting the curves using the 1:1

Langmuir binding model for the interaction between His-tagged proteins and $Ni^{2+}$-activated NTA surfaces were determined to be $1.49 \times 10^{-6}$ M and $6.05 \times 10^{-9}$ M for His-tagged HetR protein and His-tagged Cu/Zn SOD protein, respectively. Such affinities have been shown high enough for subsequent binding assays.[38] The difference in relative equilibrium immobilization levels which we observed between His-tagged Cu/Zn SOD and HetR can be partly explained by the 2-fold difference in their injection concentrations, $Ni^{2+}$ concentration, protein molecular weight, and the difference in their affinities for the $Ni^{2+}$-activated NTA surface. Such differences have been previously shown to be dependent on the microenvironment of the His-tag within the proteins.[13]

In contrast to the two His-tagged proteins, the non-tagged SOD protein shows almost no affinity for the $Ni^{2+}$-activated surface (Figure 3d), demonstrating essentially 100 % selectivity of the NHC-NTA surface for the presence of the 6×His-tag proteins. This suggests the NHC-NTA surface specifically binds the protein of interest while resisting non-specific adsorption of other proteins. For both tagged proteins, regeneration of the NHC-NTA surface was relatively simple; for example, less than 10 % of the His-tagged HetR and only 1 % of His-tagged SOD remained after regeneration treatment. Such a relatively incomplete regeneration is typical for an NTA surface, as it tends to retain a low number of His-tagged ligands.[39] Regeneration of the NHC-NTA surface by removal of the His-tagged ligand would allow multiple uses of the same NTA surface when regeneration of ligand-analyte complex is difficult. Potentially, this NHC-NTA chip offers advantages over dextran-modified chips in which the protein is directly bound to the surface in a randomly oriented fashion through amine coupling. Specifically, the NHC-NTA surface allows control of the orientation of immobilized His-tagged proteins, ensuring the active sites are accessible to the analyte.

Further tests of the NHC-NTA chip's ability to specifically bind protein were again focused on streptavidin. One drawback of the SA-based sensor surface described in the previous section is that biotinylated ligands can bind to streptavidin surface with extremely high affinity; therefore, regeneration of the streptavidin surface is commonly difficult to accomplish. Therefore, we describe a regenerable SA chip based on the NHC-NTA platform (NHC-NTA-SA chip) to potentially solve the above-mentioned problem. Importantly, its performance is essentially the same as observed on the SA chip (Figure 4).

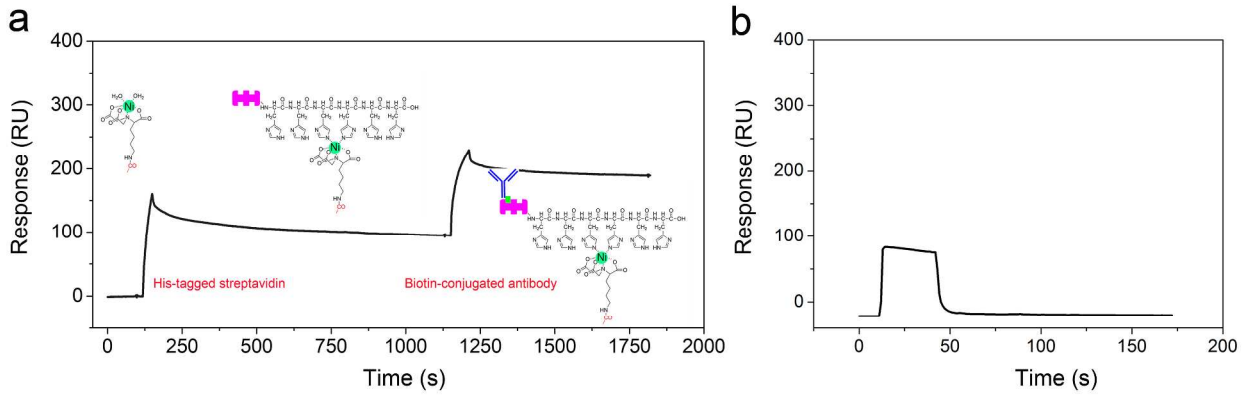


**Figure 4**. (a) The NHC-NTA surface was first exposed to the $Ni^{2+}$ solution for 2 min, followed by a 30 sec injection of His-tagged streptavidin and injection of the biotin-conjugated antibody for 1 min. (b) Control test on the NHC-NTA surface without $Ni^{2+}$ solution exposure. The amount of non-specific binding level of His-tagged streptavidin adsorbed onto the NHC-NTA surface was determined by subtracting the value of response unit prior to the protein injection from the corresponding value measured after the final 2 min buffer rinse.

The NHC-NTA surface was first exposed to the $Ni^{2+}$ solution, followed by injection of His-tagged streptavidin (Figure 4a), resulting in the NHC-NTA-SA surface with a streptavidin immobilization level equivalent to 94 RU. Then biotin-conjugated antibody (mouse anti-goat IgG-B) was injected over the NHC-NTA-SA surface, resulting in a binding signal of approximately 125 RU. A control test was also performed on the NHC-NTA surface, giving a negligible binding signal (1.5 RU) for His-tagged streptavidin without $Ni^{2+}$ solution activation

(Figure 4b). Again, this confirmed the excellent selectively of the NHC-NTA platform. This suggests that the NHC-NTA-SA surface can be used for affinity capture of the biotinylated molecules in subsequent assays, as demonstrated for the NHC-SA surface. Regeneration of this surface can be achieved by several injections of 500 mM EDTA with 500 mM imidazole by removing $Ni^{2+}$ and the chelated His-tagged streptavidin as well as the biotin-conjugated antibody. Together, these results suggest that the NHC-NTA surface can readily and rapidly capture and distinguish the affinities of His-tagged proteins and, thus, has the potential to serve as an efficient platform for studying interactions of proteins and other molecules.

### 3.3 Validation of NHC-Protein A Chip

The affinity-capture approach using a sensor surface immobilized with protein A permits controlled, reproducible capture of the antibodies by their Fc-region, producing a uniformly oriented antibody surface, which allows their antigen-specific regions to be biologically available.[21,40–42] In this study, we further test the application range of the dextran-modified NHC platform by functionalizing it with a recombinant protein A to produce an NHC-Protein A sensor surface (Figure 5).

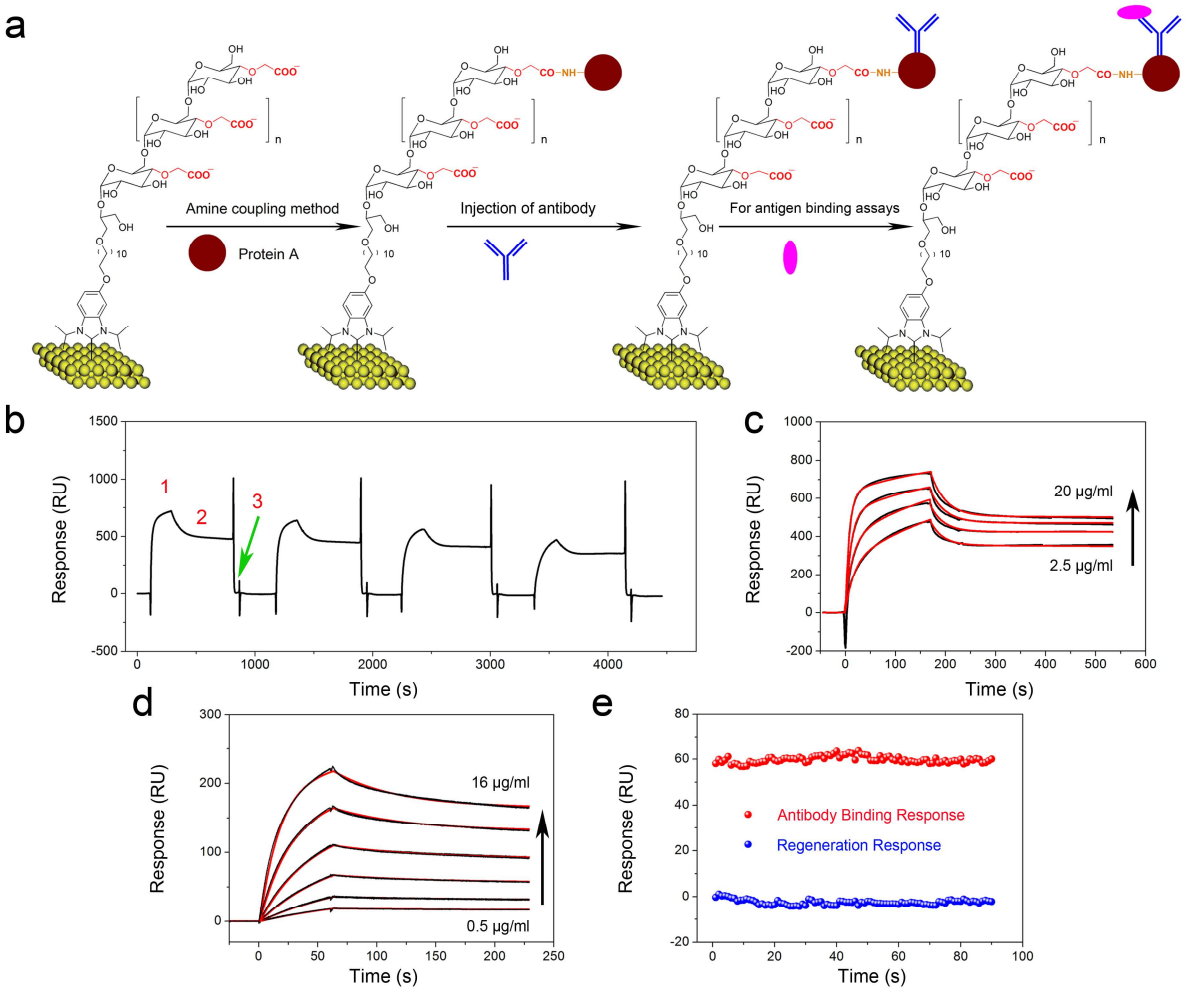


**Figure 5.** (a) Schematic illustration of the preparation procedure of NHC-Protein A surface from an NHC-supported dextran matrix for studying antibody-antigen interactions. (b) Reference-subtracted sensorgrams for serially diluted injections of normal rabbit IgG (sc-2027) antibody over an NHC-protein A surface. Regions 1, 2, and 3 correspond to antibody, buffer, and regeneration solution injections. (c) Double-referenced sensorgrams (black lines) and the corresponding fitting curves (red lines) of (c) normal rabbit IgG (sc-2027) and (d) normal mouse IgG (sc-2025) binding to NHC-protein A surfaces. (e) Binding responses of 90 binding/regeneration cycles between normal mouse IgG (sc-2025) and an NHC-Protein A surface.

Using a series of antibody injections, we tested the ability of the protein A modified NHC-supported dextran surface to act as a capture platform, resulting in the formation of a stable, antibody-terminated surface (Figure 5b). For each series of antibody binding/regeneration cycles,

the response for antibody loading on the NHC-Protein A surface approached stable levels within a few minutes following each antibody injection. For regeneration of antibody bound to the NHC-Protein A surface, a single injection of regeneration solution was sufficient to remove over 98 % of bound antibody from the NHC-Protein A surface for both antibodies over our tested range of concentrations. In addition, a control test was performed upon delivery of 50 μg/ml BSA solution over the NHC-Protein A surface, which showed no observable/minimal non-specific binding response (Figure S2). This strongly supports the excellent selectivity toward the IgG antibody for the NHC-Protein A surface.

Over the course of each series of antibody injection (Figure 5b), baseline levels were observed to show a cumulative change of less than 2 % of the response levels for antibody injections, suggesting that the NHC-Protein A surface is a highly stable platform for antibody presentation in antibody-antigen studies. The apparent ease of regenerating the antibody loading on the NHC-Protein A surface, combined with the stability of the baseline for multiple cycles of antibody immobilization would facilitate the renewal of the antibody surface if the loss of antibody-antigen activity is observed during binding studies. A series of double-referenced sensorgrams of normal rabbit IgG (sc-2027) binding to the NHC-protein A surface is shown in Figure 5c (black lines). Recent reports have shown that IgG antibody has a high tendency to undergo conformation change when binding to protein A, resulting in a reduced hydrodynamic radius and changes in secondary structure.[43,44] The use of the Langmuir 1:1 model was therefore not appropriate, and a two-state reaction model was employed to fit the data.[12–14]

$$C + D \leftrightharpoons CD \leftrightharpoons CD^{*} \quad (5)$$

This model describes a 1:1 binding between analyte and ligand, followed by a conformational change,[14,16] where $C$ representes the antibody in solution, $D$ is the free surface binding sites of protein A, $CD$ represents the surface-bound antibody-Protein A complex and $CD^*$ corresponds to the complex after conformational change. In equations 6 and 7, $k_{a1}$ and $k_{d1}$ are association and dissociation rate constants for $C$ binding to $D$, respectively, and $k_{a2}$ and $k_{d2}$ are forward and reverse rate constants for the conformational change between $CD$ and $CD^*$, respectively.

$$\frac{d[D]}{dt} = k_{d1}[CD]_t - k_a[C]_t[D]_t \quad (6)$$

$$\frac{d[CD]}{dt} = k_{a1}[C]_t[D]_t - k_{d1}[CD]_t + k_{d2}[CD^*]_t - k_{a2}[CD]_t \quad (7)$$

$$\frac{d[CD^*]}{dt} = k_{a2}[CD]_t - k_{d2}[CD^*]_t \quad (8)$$

Then $K_D$ can be derived using the following equation:

$$K_D = \frac{1}{(k_{a1}/k_{d1}) \times (1+k_{a2}/k_{d2})} \quad (9)$$

The corresponding fit curves correspond closely with sensorgram data (Figure 5c), implying that the two-state reaction model agrees well with the observed binding kinetics. The $K_D$ was calculated to be (4.63 ± 1.91) × $10^{-9}$ M, which is in good agreement with previously reported values for protein A binding to the Fc region of IgG antibody from various sources with high $K_D$ ~ $10^{-9}$ M.[21]

To test the generality of the NHC platform used in different SPR-based devices, we next constructed an NHC-Protein A chip, with a protein A immobilization level of 560 RU, on a 500 kDa NHC-CM surface for use in a SensiQ SPR platform (Pall FortéBio). We tested the binding

interactions between normal mouse IgG (sc-2025) and the NHC-protein A surface (Figure 5d). The corresponding responses show exponential trends, which are concentration dependent and reproducible. Note that different antibodies may have different affinity toward protein A. The $K_D$ obtained using the two-state reaction model was measured to be $(3.21 \pm 0.02) \times 10^{-8}$ M. Additionally, the stability of the NHC-Protein A chip was tested by 90 binding/regeneration cycles on the SensiQ SPR device (Figure 5e). An example of the corresponding 10 binding-regeneration cycles is shown in Figure S3. These results show that the binding responses and binding curvatures of consecutive antibody injections are highly consistent throughout the 90 binding cycles, without any noticeable decay detected in the antibody binding, further demonstrating the robust feature of the NHC-Protein A surface and the suitability of the NHC-based platform to be employed in SPR systems.

## CONCLUSIONS

In conclusion, we have demonstrated the practicality of NHC-supported dextran surface as a functionally versatile biosensing platform for evaluation of biomolecular interactions with high selectively and robust performance and stability. Each of the surfaces tested (NHC-SA, NHC-NTA, and NHC-Protein A) utilized the incorporation of a functional group to enable the non-covalent capture of a ligand with high purity and consistent orientation, thus maximizing the potential binding activity of the biosensor surface for the analyte of interest. SPR tests of the NHC-SA surface showed low non-specific binding in the aptamer screening test, and nearly identical performance to a similarly derivatized commercial thiol-based chip in a binding kinetics experiment. Tests of the NHC-NTA surface showed the ability to discriminate between His-tagged and non-His-tagged proteins, and facile regeneration with baseline stability. NHC-Protein A chip tests showed good antibody binding response with rapid attainment of equilibrium

binding levels and baseline stability between subsequent binding/regeneration cycles. In addition to the enhanced stability of the NHC SAMs demonstrated in previous studies,[4,5,9,10] our tests collectively show the promise of the NHC-CM surface as a practical and versatile biosensor platform. Taken together, these results validate the prospective strategy for developing versatile biosensor surfaces based on NHC chemistry, highlighting the flexibility of surface design and facilitating potential integration with other state-of-the-art techniques, such as optical, thermal optical, piezoelectric, acoustic wave, and electrochemical techniques.

## ASSOCIATED CONTENT

**Supporting Information**. This material is available free of charge via the Internet at http://pubs.acs.org.

Chemicals and materials. Preparation procedure for dextran-modified NHC surfaces and an extended data set for biotin binding, non-specific protein binding, binding-regeneration cycles, aptamer sequences and the corresponding binding levels.

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