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N-Heterocyclic Carbene Self-Assembled Monolayers on Gold as Surface Plasmon Resonance Biosensors

Zhijun Li,† Kim Munro,‡ Iraklli I. Ebralize,† Mina R. Narouz,† J. Daniel Padmos,† Hongxia Hao,§ Cathleen M. Crudden,†¶ and J. Hugh Horton†*

† Department of Chemistry, Queen's University, Kingston, Ontario, K7L 3N6, Canada

‡ Protein Function Discovery Facility, Queen's University, Kingston, Ontario, K7L 3N6, Canada

§ Collaborative Innovation Center of Judicial Civilization and Key Laboratory of Evidence Science, China University of Political Science and Law, Beijing, 100088, China

¶Institute for Transformative Bio-Molecules (ITbM-WPI), Nagoya University, Chikusa, Nagoya, 464-8602, Japan

ABSTRACT: Surface plasmon resonance (SPR)-based biosensing is a powerful tool to study the recognition processes between biomolecules in real-time without need for labels. The use of thiol chemistry is a critical component in surface functionalization of various SPR biosensor surfaces on gold. However, its use is hampered by the high propensity for oxidation of the gold-thiol linkage even in ambient atmosphere, resulting in a short lifetime of SPR sensor chips unless strict precautions are taken. Herein, we describe an approach to overcome this limitation by employing highly robust self-assembled monolayers (SAMs) of alkylated N-heterocyclic carbenes (NHCs) on gold. An alkylated NHC sensor surface was developed and its biosensing capabilities were compared to a commercial thiol-based analogue – a hydrophobic association (HPA) chip – in terms of its ability to act as a reliable platform for bio-specific interaction analysis under a wide range of conditions. The NHC-based SPR sensor outperforms related thiol-based sesnsors in several aspects, including lower non-specific binding capacity, better chemical stability, higher reproducibility, shorter equilibration time, and longer life span. We also demonstrate that the NHC-based sensor can be used for rapid and efficient formation of a hybrid lipid bilayer for use in membrane interaction studies. Overall, this work identifies the great promise in designing NHC-based surfaces as a new technology platform for SPR-based biosensing.

Surface plasmon resonance (SPR) biosensing is a label-free and highly sensitive optical technique that can be used to probe biomolecular interactions in real-time.[1–4] In a typical SPR-based biosensing experiment, a solution of analyte flows through a microfluidic array across a planar gold surface, onto which an appropriate ligand has been immobilized.[1] As the analyte binds to the ligand, it alters the refractive index of the medium close to the metal surface; subsequent changes to the maximum reflectance angle at which light is absorbed may then be used to determine the quantity of analyte adsorbed to the surface in real-time.[3] Thus, label-free detection of biomolecules may be achieved, as well as direct measurement of ligand-analyte binding affinity and kinetics.[3] To date, the SPR-based biosensing technique has become essential in pharmaceutical and life sciences research.[3,4]

Commercial SPR instruments usually rely on gold surfaces because, as a metal, Au is resistant to oxidation, biocompatible, and exhibits intense SPR signals.[4] However, an unmodified gold surface has a high tendency to spontaneously adsorb biomolecules, and many proteins undergo denaturation or random orientation upon binding to gold, resulting in unreliable assays.[5,6] Therefore, tremendous efforts have been geared towards the development of functionalized gold surfaces for use in SPR-based biosensors to detect bio-analytes in solution and enable the study of a wide range of biomolecular interactions.[7,8] In such applications, gold is typically functionalized with thiol-based self-assembled monolayers (SAMs) to which various tail groups are attached, tailored to react specifically with the target biomolecules to be studied.[8–10]

Many SPR studies are carried out with an alkanethiol-based sensor. Commercial versions of such sensors are available:[4] two such being the hydrophobic association (HPA) chip or a lipophilic vesicle capture (L1) sensor chip.[11–13] The HPA chip consists of octadecanethiol covalently bound to a gold surface.[4,11] A lipid monolayer may then be self-assembled onto this surface, in which the lipid molecules interdigitate with the alkyl chains of the alkanethiol SAM by hydrophobic interactions to form a supported hybrid lipid bilayer.[14] An alternative to the HPA chip, the L1 chip is composed of a thin dextran layer derivatized by lipophilic groups on a gold surface.[12] In both cases the formation of a hybrid lipid bilayer has been shown to block non-specific protein adsorption and to mimic membrane surfaces for studies of signal transduc-

tions by SPR in membrane-like environments.[11,12] Such membrane interfaces are important for diverse biological processes that involve the association of receptors and ligands at the interface.[14–17]

Unfortunately, the thiol-based SAMs used to create these biosensors display limited physical and chemical stability because the sulfur headgroups have a high propensity for oxidation.[18,19] Therefore, the potential utility of thiol-based SAMs for wide applications are hampered unless strict precautions are taken, such as limiting exposure in air to a short period or storage in an inert atmosphere or under solution to slow down the oxidation process.[19] Commercially available sensor chips are packaged in a nitrogen atmosphere and have a shelf life of approximately 3 months at 4 °C.[20,21] Although various strategies have been developed to improve the stability of thiol SAMs such as adopting polydentate thiols or increasing the intermolecular interactions, the development of a more stable chemical linker to gold is the most direct solution.[9,19,22–27]

The use of N-heterocyclic carbenes (NHCs) to functionalize gold surfaces has received significant attention as NHCs have the potential to overcome many obstacles in gold-thiol chemistry.[22–30] As excellent ligands for transition metals, NHCs have risen from academic curiosities to commercially available compounds in various applications.[23] This remarkable stability results from the overall electronic and steric contributions of the special structural features of NHCs. We have previously reported NHC-based SAMs on gold that exhibit excellent stability over a wide range of harsh conditions.[24] Like thiols, these NHCs may be synthesized with a wide range of functional groups on the pendant side chain. This attractive structural versatility is a key strength for NHCs as potential candidates in surface functionalization by introducing optical, electrical, chemical, and biochemical properties.[25]

More recently, we have developed imidazolium hydrogen carbonates which are bench-stable solid precursors that can be used to prepare NHC SAMs under ambient conditions, along with preliminary results of the potential applications in SPR biosensing.[26] Here, we report a detailed study of characterization and evaluation of NHC-based biosensor chips (NHC-chips) compared to commercial thiol analogues (HPA-chips). The performance of the NHC chip with regard to lipid monolayer formation was compared to that of the HPA chip in terms of the hybrid lipid bilayer characteristics, reproducibility in biosensing, and stability over a wide range of experimental conditions, as well as its efficacy in probing a peptide/lipid membrane interaction. We expect this new approach to the formation of planar-based sensor surfaces will greatly impact the design of SPR-based devices and expand the range of available biosensing applications.

EXPERIMENTAL SECTION

Preparation of NHC Sensor Surfaces. Gold surfaces (SIA kit Au, GE Healthcare) were stored at 4 °C. The sensor surfaces were first cleaned by immersion in a mixture of NH_4OH: H_2O_2: H_2O (1:1:5) at 80 °C for 0.5 h (caution: strong oxidizing agents), rinsed with milliQ-water, and dried under a stream of nitrogen. A plasma cleaning step was performed for 15 min with a Harrick Plasma Cleaner/Sterilizer (PDG-32G). The NHC precursors (5-(dodecyloxy)-1,3-diisopropyl-1H-benzo[d]imidazol-3-ium hydrogen carbonate) [1] and (5-((11-hydroxyundecyl)oxy)-1,3-diisopropyl-1H-benzo[d]imidazol-3-ium hydrogen carbonate) [2] were used. Complete details on the synthesis and characterization of these species may be found elsewhere.[26] The gold surfaces were then immersed into a 10 mM solution of the corresponding NHC precursors in dry methanol for 48 h at room temperature (without light). Finally, the sensor surfaces were rinsed with methanol, Milli-Q water, dried under nitrogen, and mounted onto a support (SIA kit Au, GE Healthcare). All biosensing tests were carried out on a Biacore 3000 SPR system (GE Healthcare). Equilibrium tests were carried out on the prepared or commercial chips using phosphate buffered saline (PBS, pH 7.4) after the sensor chip was docked into the SPR system and primed with the running buffer. Sensor chips were typically stored at 4 ºC in 50 ml centrifuge tubes with a small portion of moist tissue for maintaining a hydration environment, for up to 9 months. Additionally, both HPA and NHC chips were subjected to a series of thermal exposures up to 65 ºC for 24 h in air. XPS and contact angle measurements were carried out as described in the Supporting Information.

Non-Specific Protein Adsorption on NHC Surfaces. Proteins used in the present study consisted of lysozyme (14 kDa, *p*I = 11.35), ovalbumin (45 kDa, *p*I = 4.5), BSA (66 kDa, pI = 5.3), concanavalin A (102 kDa, *p*I = 4.5–5.5), and fibrinogen (340 kDa, *p*I = 5.5). Each protein was dissolved in PBS buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4) at a concentration of 0.1 mg/ml. The protocol for measuring the non-specific adsorption of protein is as follows: after initial baseline equilibration, the PBS buffer was flowed over the sensor surface for 2 min followed by an injection of a protein solution for 3 min, and finally by a 5-min buffer rinse. The flow rate was maintained at 10 µl/min throughout these tests. Regeneration of the sensor surfaces was effected by one 1-min injection of 40 mM n-Octyl β-D-glucopyranoside (OG) followed by one 1-min injection of 50 mM NaOH. The amount of protein adsorbed onto the surface was determined by subtracting the value of response unit prior to the protein injection from the corresponding value measured after the final 5 min buffer rinse.

Formation and Stability Tests of Hybrid Lipid Bilayers on HPA and NHC Chips. L-α-Phosphatidylcholine from egg yolk (9.0 mg, 0.012 mM) was used to prepare small unilamellar vesicles (SUVs) for formation of hybrid lipid bilayer on the SPR chips. The lipid was first dissolved in chloroform/methanol (2/1, v/v) followed by addition of PBS buffer to afford a 2 mM suspension and the vial was vortexed until a milky suspension was formed. Then a freeze–thaw procedure was repeated for 8 cycles, consisting of an 8-min freeze in dry ice/acetone, followed by an 8-min thaw in hot water at 80 °C. Sonication was performed to give a translucent suspension of SUVs with a predomi-

nant size range between 30 and 35 nm as determined by transmission electron microscopy (TEM). The sensor surface was first conditioned by an injection of OG. SUVs were injected for 25 min, followed by a 5-min dissociation process with buffer. Then, high flow rate buffer wash and sodium hydroxide solution were used to remove any loosely bound vesicles. The degree of lipid layer coverage of the sensor chip was evaluated by the amount of lipid bound and BSA bound to assess the non-specific protein adsorption. After each binding cycle, the sensor surface was regenerated by an injection of OG. The stabilities of the SUV-based lipid monolayers formed on HPA and NHC chips were studied by a similar approach as reported by Cooper *et al.*,[11] for 3 h in PBS buffer followed by a 2-min (10 µl/min) exposure to a series of regeneration solutions chosen to evaluate the chips' response to extremes of pH and ionic strength.

Mellitin Binding Tests. SUVs of egg phosphatidylcholine/cholesterol (10:1 w/w) were prepared as described above in PBS buffer (20 mM Na_2HPO_4/NaH_2PO_4, pH 6.8). The resultant lipid monolayers formed on the sensor surfaces were used as a model cell membrane to scrutinize peptide-membrane interactions.[31] Melittin was diluted twofold in PBS buffer from 0.3 to 0.014 µM and passed serially at a flow rate of 10 µl/min over the lipid surface for 4 min. Then it was replaced by PBS buffer to allow the lipid-melittin complex to dissociate for 6 min. Regeneration of the surface was effected by an injection of 10 mM NaOH.

RESULTS AND DISCUSSION

Characterization of NHC Surfaces. SAMs are formed from solution by adsorption of organic molecules onto a solid surface, rearranging into highly ordered and oriented monolayers with fine chemical control at the molecular level.[32–34] Figure 1 shows a schematic illustration of the commercial HPA chip, and the NHC chips examined here. The commercial HPA chip is formed by octadecanethiol covalently bound to a gold surface,[4] whereas the NHC chips fabricated by us were prepared by self-assembly of [1] or [2], both alkylated NHC imidazolium hydrogen carbonate salts.[26]

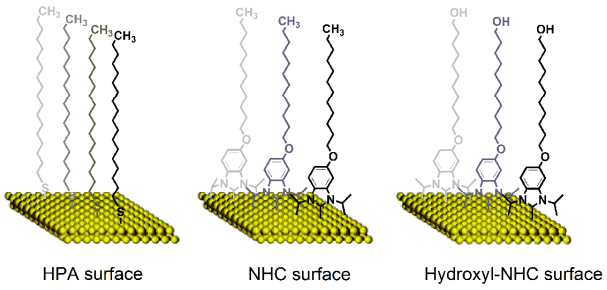

Figure 1. Schematic illustrations of HPA surface (SAM formed from octadecanethiol); NHC surface (SAM formed from [1]) and hydroxyl-NHC surface (SAM formed from [2]).

Here, NHC molecule [1] or [2] was self-assembled onto the gold surface. The alkylated NHC surface was used to compare with the corresponding thiol analogue (HPA chip) for use in model membrane studies. The hydroxyl-NHC surface was employed to support the evidence of the general wettability of the NHC SAMs. An extensive report on the surface characterization of SAMs prepared from [1] may be found elsewhere.[24,26]

Octadecanethiol on gold (i.e., the HPA chips by Biacore (GE Healthcare)) have been widely studied and the corresponding XPS analysis can be found in previously published literature.[32–34] The surface chemical composition of the NHC surface prepared from [1] was evaluated using X-ray photoelectron spectroscopy (XPS) and the corresponding spectra are shown in Figure S1. An N 1s peak is observed at 400.6 eV indicating the presence of the NHC on the gold surface.[22,28] The C 1s spectrum deconvolutes into two components: one at 284.9 eV represents aromatic carbon, whereas that at 286.4 eV is attributed to the aliphatic carbon of the alkylated NHC molecule. The observed C:N ratio is 26:2 which is close to the stoichiometric C:N ratio of the molecule (25:2). This confirms that the NHC molecule deposited cleanly on the gold surface. The XPS data for the hydroxyl-NHC surface is summarized in supporting information (Figure S2).

In order to macroscopically study the structure and chemistry of the material surfaces, surface wettability measurements were performed using Milli-Q water as the contacting liquid.[33] The static contact angle (CA) on an unmodified gold surface is 86 ± 5 °. That of octadecanethiol on gold is 112 °.[33,34] The alkylated NHC surface prepared from [1] has a less hydrophobic CA of 78 ± 3 °. This is consistent with the relatively lower packing density of the alkylated NHC SAMs (3.5 molecules/nm^2) compared to the densely-packed alkyl thiols (4.6 molecules/nm^2) on gold surfaces.[24,26,35] This would allow increased atomic contacts between water and methylene groups of the NHC SAMs.[36] In contrast, for the more densely packed alkyl thiol SAMs, the water interacts predominantly with the terminal methyl groups rather than the underlying methylene groups.[36] This is the reason that leads to the different contact angles. As will be discussed later, this difference in the hydrophobic characteristics of the NHC surface may give rise to different interfacial properties in biosensing applications as compared to the thiol analogue (HPA chip). A hydroxyl-terminated NHC was also prepared from [2] (see Figure 1) to elucidate the effect of wettability on non-specific protein adsorption of NHC-based surfaces. The CA of hydroxyl-terminated NHC surface is 68 ± 3 °, considerably less hydrophilic than hydroxyl-terminated thiol SAMs which typically exhibit a CA less than 15 °.[8,33] Again, this likely results from the difference in the packing densities between the NHC and thiol, which lead to the interactions between the water and the underlying methylene groups in the hydroxyl-terminated NHC SAM, as well as the presence of the hydrophobic isopropyl groups.

In order to more fully characterize the difference in surface properties in relation to non-specific adsorption, SPR was used to quantify the adsorption of various proteins on the two NHC surfaces in PBS buffer (Figure S3). Results are

summarized in Table S1. Lysozyme is a small molecular weight protein (14 kDa, *p*I = 11.35) that is positively charged in PBS buffer at pH 7.4, and is commonly used in studies of electrostatic adsorption of proteins to surfaces.[37,38] Ovalbumin, bovine serum albumin (BSA), and concanavalin A are medium molecular weight proteins which are negatively charged in PBS buffer. Fibrinogen (340 kDa) is a large protein that is known to adsorb strongly to hydrophobic surfaces.[38]

Notably, the two most hydrophobic proteins (BSA and fibrinogen) are adsorbed to the alkylated NHC surface to a significantly greater degree than that on the hydroxyl-terminated NHC surface, consistent with observations on the corresponding thiol-based surfaces previously reported by Whitesides *et al.*.[37] The amount of lysozyme adsorbed on the alkylated NHC surface is more than twice that observed on the hydroxyl-terminated NHC surface. This suggests that the lysozyme favors hydrophobic interaction with the alkylated NHC surface over the more hydrophilic hydroxyl-terminated NHC surface.[37] However, there is still a higher amount of lysozyme adsorbed on the hydroxyl-terminated NHC surface compared to the reported data on hydroxyl-terminated thiol SAMs.[37,38] This phenomenon is consistent with the CA tests that implied the hydroxyl-NHC surface is more hydrophobic than its thiol analogue. Ovalbumin and concanavalin A show little difference in selectivity between the two surfaces.

Equilibrium tests on HPA and NHC chips. Equilibrium tests were carried out on 4 flow cells over the HPA and alkylated NHC chips using PBS buffer, as shown in Figure 2. Although extreme care (thoroughly cleaned glassware, buffer, flow rate, temperature) was taken to minimize the baseline drifts,[39] obvious upward baseline drifts on 4 flow cells (FCs) over the HPA chip are observed. This resulted in a waiting period of at least 10 h for the HPA chip to come to equilibrium in the PBS buffer. By contrast, only 1.5 h is needed for the NHC chip to come equilibrium and be ready to use for subsequent biosensing assays. There are approximately 1700 RU and 120 RU (Response Unit: 1 RU corresponding to a 0.0001 ° angle shift in SPR response) maximum signal differences distributed over four flow cells for the HPA and NHC chips, respectively. The smaller signal difference indicates a relatively higher quality and homogeneous coating system.[4] These results suggest that NHC-based surfaces are significantly superior in respect to equilibration times and sample-to-sample reproducibility; the reasons for this performance difference are examined more closely in subsequent experiments.

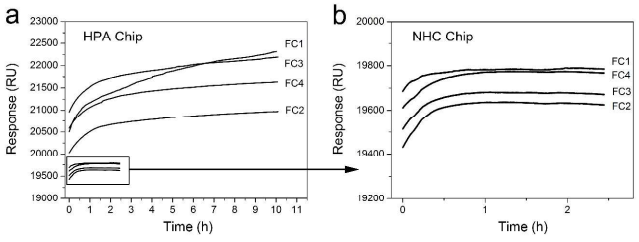

Figure 2. Equilibrium tests on 4 flow channels (FC1 through FC4) over (a) HPA and (b) alkylated NHC chips in PBS buffer. The inset in (a) is the equilibrium test on the NHC chip at the same scale. Note that FC1, as the lead channel, exhibits differing behavior due to continuous exposure to buffer.

Formation and Regeneration of Hybrid Lipid Bilayers. The primary use of alkylated SPR chips such as the commercial HPA is to act as a support for the formation of a model membrane surfaces that themselves may then be used to study further biochemical interactions. Here, we report on one such hybrid lipid bilayer, formed by the adsorption of phosphatidylcholine lipid vesicles onto the HPA or NHC SPR sensor chips. Neutral phosphatidylcholine lipid is commonly used in such membrane studies because negatively charged lipids affect the coverage of lipid layers when fused on the sensor surface.[40] Here, we use neutral egg yolk L-α-phosphatidylcholine (egg PC) for this purpose. A TEM image of small unilamellar vesicles (SUVs) prepared from the egg PC have a predominant size of 30 ~ 35 nm, as shown in Figure S4. These vesicles are then adsorbed onto the sensor chip surface. Having adsorbed the vesicles, they must also be removed following any given experiment. Extensive regeneration experiments were carried out to this effect, and are described in more detail in the following section. However, for the purposes of the experiments described here, nonionic n-octyl β-D-glucopyranoside (OG) was used to remove the lipid layer, in addition to removing any proteins, allowing the surface to be cleaned and reused.[3,11] Therefore, we tested a wide range of OG concentrations to assess their ability to remove lipid from the NHC surface (known as a regeneration scouting step) to enable consistent lipid binding performance for assays. Similar to results on an HPA chip,[11] we found 40 mM OG to be optimal for regenerating the alkylated NHC chip in the assays described here.

Figure 3 shows SPR sensorgrams tracing the formation of hybrid lipid bilayers on the HPA and NHC chips in Citrate buffer (pH 5.0). Prior to injection of lipid vesicles, the surfaces were conditioned with an injection of 40 mM OG, as noted above. The resulting change in solution refractive index may be seen as an increase followed by a decrease in SPR response back to the baseline between t = 50 – 350 s. The lipid vesicles were then injected at t = 560 s, and allowed to adsorb onto the surface. Following the injection of vesicles, the surfaces were subjected to a dissociation phase using buffer alone and then exposed to a high flow-rate buffer wash and an injection of NaOH, at t =

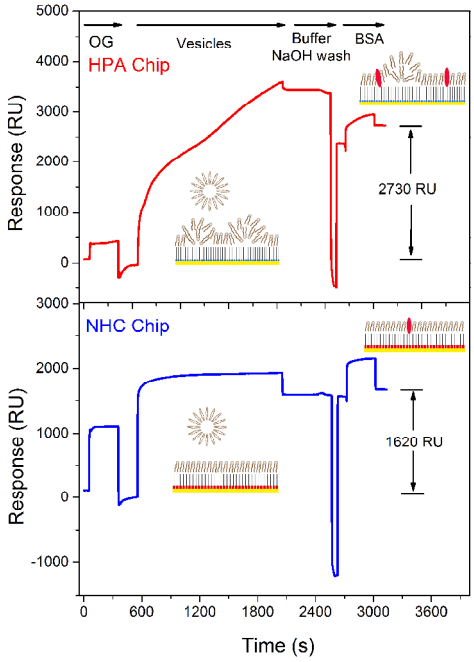

Figure 3. Formation of lipid monolayers on HPA (red) and NHC (blue) chips in Citrate buffer (pH 5.0) by successive injections of 40 mM OG, 2 mM SUVs, 50 mM NaOH, and 0.1 mg/ml BSA over the sensor surfaces.

2420 s, to remove any loosely bound vesicles. Again, these steps resulted in transient changes in response due to changes in the solution refractive index. Comparing the sensorgram characteristics of lipid association on the HPA and NHC surfaces, two principle differences can be observed. First, saturation of the sensor surface during the injection of lipid vesicles (t = 560 to 2100 s) was approached in a much shorter time-span for the NHC chip as compared to the HPA chip. Second, the level of lipid association during vesicle injection was almost two-fold higher for the HPA chip than that of the NHC chip, while a greater amount of the lipid associated with the HPA chip surface was removed by the subsequent high flow-rate buffer and NaOH washes. This suggests that a large proportion of the associated lipid was not incorporated into the stable lipid monolayer on the HPA chip surface as compared to the NHC chip. Furthermore, the material that remains on the HPA surface is greater: the post-wash levels of the associated lipid at the conclusion of the sensorgram cycle (2690 s) are higher for the HPA chip (2361 RU) than that of the NHC chip (1507 RU). In a second stage of the experiment, the quality of the lipid overlayer was probed, through exposure to a solution of the highly hydrophobic protein BSA at t = 2700 s through 3000 s. Since BSA is not a membrane-bound protein but instead binds non-specifically to the underlying hydrophobic SAM, BSA adsorption indicates gaps or defects in the supported hybrid lipid bilayer.[11,13] Less BSA (113 RU) was adsorbed on the NHC chip compared to that (369 RU) on the HPA chip in the experiment shown in Figure 3; similar results were obtained from an average of eight measurement cycles, indicating that the lipid overlayer was indeed more continuous on the NHC chip.

Further confirmation was obtained by comparison of SEM images of the lipid layers formed on HPA and NHC surfaces in Citrate buffer (Figure 4). The SEM image of the HPA-supported lipid surface shows the presence of numerous lipid vesicles, many of which remain on the surface, even following the NaOH conditioning step. By contrast, the lipid deposited on the NHC surface appears to be uniform before and after conditioning, resulting in the formation of a supported hybrid lipid bilayer, consisting of interdigitated alkyl groups from the surface and the hydrophobic end groups of the lipid. The hydroxyl-terminated NHC surface cannot support a lipid layer due to its hydrophilicity and therefore no SEM was applied here. Being able to support such a layer is an important characteristic of any planar SPR system that is employed as a realistic model for evaluating the binding interactions of membrane-associated proteins.[11,15,16]

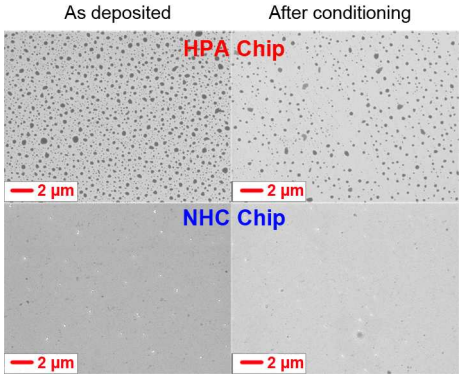

Figure 4. SEM images of lipid deposited on HPA and NHC chips in Citrate buffer before and after conditioning.

Formation of similar hybrid lipid bilayers is also reproducible in a number of other buffer systems, and following multiple formation and removal cycles. Figure 5 shows the sensorgrams of repeated cycles of lipid layer formation and regeneration on the HPA and NHC chips in PBS (pH 7.4) and Citrate (pH 5.0) buffers. With the NHC chip, a similarly high level of consistency in the character of the kinetic profiles was observed. Notably, the amount of non-specific BSA adsorption was lower and far more reproducible from run to run on the NHC chip in both buffers than it was for the thiol-based HPA chip. A similar degree of reproducibility in the lipid layer formation on the NHC chip was also achieved under a wide range of buffers varying from pH 5.0 to 10.0 (Supporting Information, Figures S5–S7, Table S2).

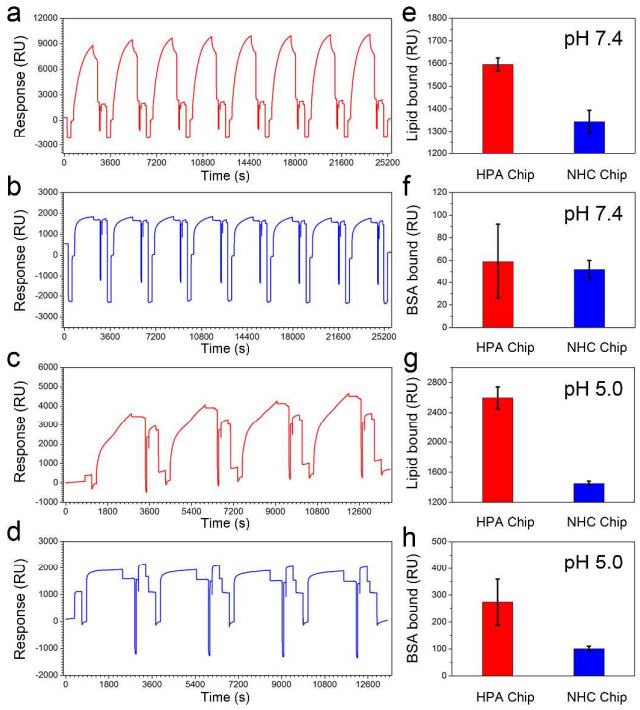

Figure 5. Repeatability of lipid binding and BSA binding on HPA (red) and NHC (blue) chips in PBS buffer (pH 7.4) for 8 cycles (a,b), and Citrate buffer (pH 5.0) for 4 cycles (c,d). The bar graphs summarize the average and standard deviation of the corresponding lipid binding and BSA binding values under the two pH regimes (e through h).

Unlike the NHC chip, the HPA chip demonstrated multilayer adsorption of lipid, regardless of pH environment (Figure 5 and Figures S5–S7). Saturation of the SPR response signal cannot be observed over the timelines used, presumably due to the multilayer adsorption phenomenon.[4] This is particularly notable at extremes of pH. For example, at pH 10.0 (CAPS buffer, Figure S7, Table S2) both an overall low amount of lipid adsorption and a much higher degree of BSA adsorption was observed on the HPA chip compared to that on the NHC version, indicative that no continuous/complete supported lipid monolayers were formed.

The failure to form complete lipid overlayers on thiol-based SAMs has some precedent. For example, Plant *et al.* investigated interactions of palmitoyl-oleoyl-phosphatidylcholine (POPC) vesicles with hydrophobic surfaces which contained methyl-terminated thiols and fluorinated thiol on gold by SPR.[41] They found the saturation of hybrid bilayer formation was far from complete after 3 h incubation. Wenzl *et al.* reported the saturation of POPC vesicles adsorbed on 1,2-dipalmitoyl-sn-glycero-3-phosphoric acid monolayer was not complete even after 30 h incubation.[42] It has been reported that the thermodynamic driving force for the formation of a hybrid bilayer is the increased entropy resulting from exclusion of water between the hydrophobic alkanethiol SAM and lipid chains.[14] The corresponding kinetics thus largely depend on the structure of the underlying SAMs.[41] The lipid adsorption behavior on the NHC chip indicates a fast saturation process for lipid hybrid bilayer formation. This differing behavior between NHC and thiol-based chips is likely due to the lower density of the alkyl chains (3.5 molecules/nm^2) compared to that of a thiol-based film (4.6 molecules/nm^2).[24,26,35] These observations highlight the potential of the NHC chip in supporting the fast and efficient formation of a high-quality hybrid lipid bilayer under a wide range of pH conditions that may be used to mimic membranes in biosensing studies.

Regeneration and Long-Term Stability. One significant weakness of alkanethiol-based SAMs is their limited thermal and chemical stability. For example, alkanethiols desorb from gold upon heating in air above 130 °C and can decompose in solvents such as hexadecane at 80 °C. Oxidative degradation of thiol-based SAMs may also be significant, occurring within lifetimes of days to weeks.[27,43] These stability limitations present challenges for achieving stable SAMs for use in SPR technology. Consequently, we compared the stability and reproducibility of the NHC-based chip to the commercial alkanethiol HPA chip in supporting the formation of the hybrid lipid bilayers described in the previous section.

As a first test, we exposed HPA and NHC SPR chips, on which the phosphatidylcholine-based hybrid lipid bilayer had been assembled, to a variety of regeneration solutions that are commonly used to disrupt ligand-analyte complexes. The baseline drift of the resulting regenerated surface was then monitored. Six regeneration solutions with different chemical properties, alone or in combination, including high ionic strength, pH extremes, and mixed regeneration solutions were used (Figure 6 and Table S3). Over the monitoring period, the HPA-based chip was found to have average drift rates less than ± 0.3 RU/min under four of the six conditions studied. The baseline drifts of the NHC-supported lipid monolayer under all six conditions were observed to be less than ± 0.3 RU/min, either similar to or considerably lower than those observed on the HPA chip. These are considered acceptable drift rates on a commercial system such as the Biacore 3000 used here.[44]

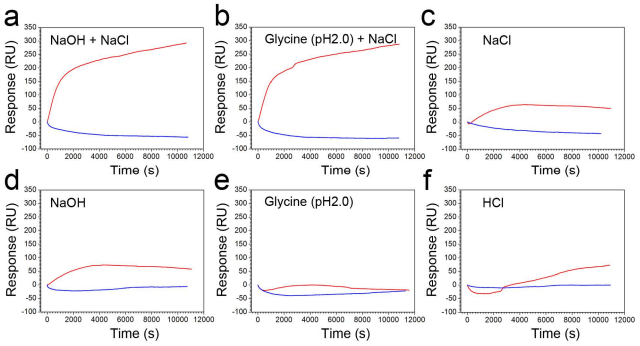

Figure 6. Stability of lipid monolayers on HPA (red) and NHC (blue) chips subject to common regeneration solutions.

Thermal stability measurements were also carried out. Here, the NHC chip was assessed by forming the hybrid lipid bilayer in PBS buffer of the NHC chip at 65 °C in air for

24 h (Figure 7 and Table S4). Equivalent data from the HPA chip are not shown here, as we found that the chip functionality was completely destroyed under such conditions. The kinetic profiles and binding results show that the lipid binding and non-specific protein adsorption on the NHC chip are essentially identical following the thermal exposure process, suggesting a robust thermal stability of the NHC chip for the formation of a lipid monolayer. Such stability implies that these chips may be used and stored over a broad range of conditions while maintaining functionality, and it could be used for thermodynamic analyses of biomolecular interactions through obtaining affinities and kinetics over wider temperature ranges than can be readily done with thiol-based systems.[3,4,45] Moreover, we envision that there might be other intriguing potential applications for the NHCs-linked gold nanoparticles. For example, the NHCs-functionalized gold nanoparticles may have uniqueness in SERS applications, particularly the nanoparticle assemblies.[46]

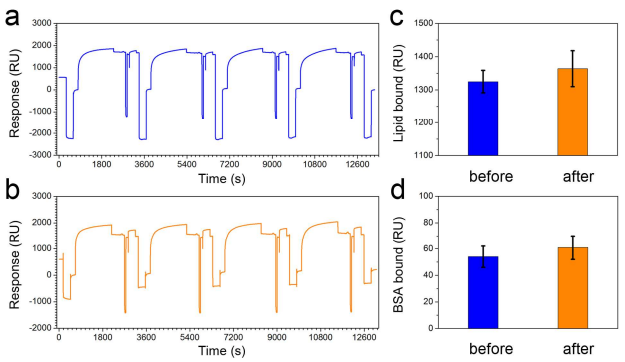

Figure 7. Sensorgrams of thermal stability test on NHC chip for 4 cycles of lipid binding and BSA binding in PBS buffer before (a, blue) and after (b, orange) thermal exposure at 65 ºC in oven for 24 h. The bar graphs summarize the average and standard deviation of the corresponding lipid binding (c) and BSA binding data (d).

To demonstrate the long-term stability of the NHC sensor surface, we carried out the lipid formation test on an NHC chip which was stored at 4 °C in air for 9 months (Figure S8 and Table S5). The result shows that the lipid monolayer formation and non-specific protein adsorption behavior on the NHC chip are essentially indistinguishable following the long-term storage. This is in contrast to the commercially available HPA chip, in which we observed a significant change in lipid adsorption behavior after fewer than 3 months' storage under identical conditions as the NHC chip.

Peptide/Lipid Interactions on HPA and NHC Chips. To provide a validation of the functionality of NHC-supported hybrid lipid bilayer, we have chosen to use it to monitor the adsorption/desorption behavior between it and the peptide melittin, which is known to form transmembrane complexes in lipid bilayers. This system was chosen due to the fact that it has been previously well-characterized on an HPA chip.[31] Egg phosphatidylcholine/cholesterol vesicles were first loaded onto the hydrophobic surfaces of the HPA and NHC chips, much as was described above. This process may be observed through the first hour of the sensogram traces shown in Figure 8, and as before the total quantity of lipid immobiled is consistent with lower quality hybrid lipid bilayer formation on the HPA chip as compared to the HPA chips. Due to the fluid impact from cholesterol in the vesicles,[47] the equilibration of the HPA-supported lipid layer requires approximately 4.8 h before the drift rate is sufficiently stable to initiate the binding assays. By contrast, the equilibration for the NHC-supported lipid layer needs less than 0.5 h. Again, it is clear that the NHC chip favors a faster vesicle fusion and a complete lipid monolayer formation, which is preferable for the fast detection of membrane-related interactions.

The resultant lipid layers were then used to compare their performance by assessing the peptide-membrane interactions. Following buffer equilibration, the lipid layer was exposed to a series of melittin solutions. These are seen as the sequential adsorption/desorption cycles following the label "Beginning of the assay" in Figures 8 a,b. Figures 8 c,d show one such cycle at the highest melittin concentration of 0.3 μM, while the Figures 8 e,f show the corresponding steady-state fitting result, obtained to determine the mellitin equilibrium affinity constant, as described in more detail below. The sudden jump in response seen in the sensorgrams of both sensor chips at t = 0 s in the inset do not correspond to binding events; instead, they are the result of mismatches in refractive index between the peptide sample and running buffer.[4] As the melittin-lipid complex forms, the refractive index changes more gradually as the mass of mellitin is accumulated on the sensor surface, resulting in an increase in response units. For melittin interactions on the NHC chip, the binding responses of each concentration approached equilibrium within 240 s. In contrast, equilibrium could not be achieved within a similar timeframe on the HPA chip, even at the highest melittin concentration (0.3 μM).

As previously discussed, we observed that an incomplete lipid layer is formed on the HPA chip surface; thus, the melittin may bind not only to the lipid layer but also to alkanethiols of the HPA chip surface through hydrophobic interaction, as shown schematically in Figure 9. By contrast, the sensorgrams

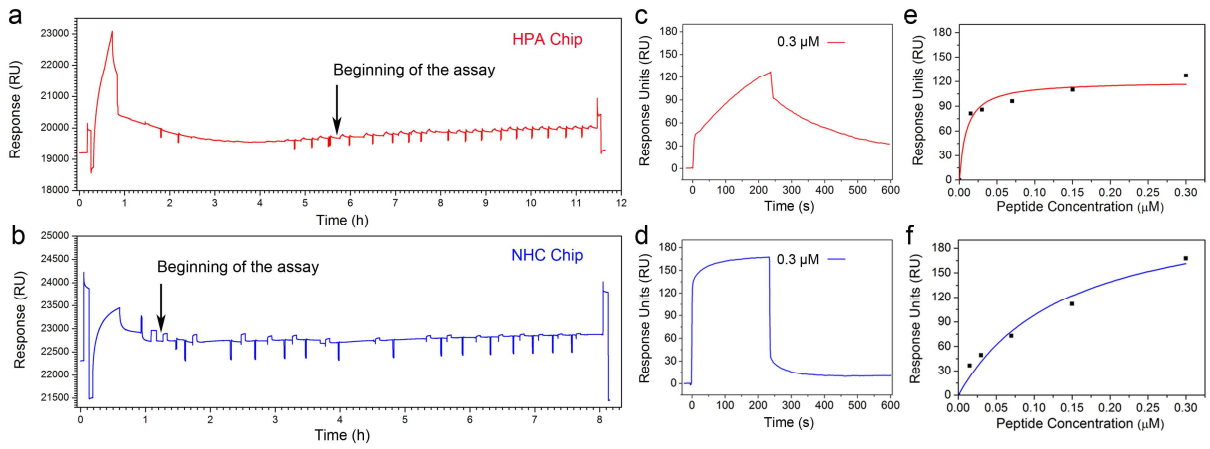

Figure 8. Experimental processes of melittin membrane interactions on (a) HPA and (b) NHC chips. The left-hand insets on both panels show one cycle of melittin binding at a concentration of 0.3 μM; the right-hand inset shows the steady-state fitting result.

indicate that the interactions were well behaved on the NHC chip, which showed a rapid approach to equilibrium and strongly concentration-dependent equilibrium binding curves as compared to the HPA chip.[48]

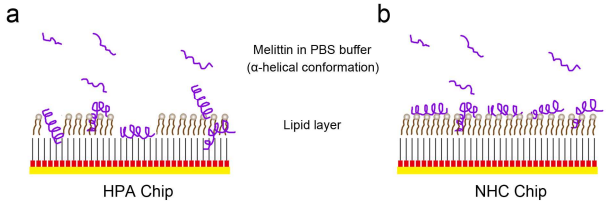

Figure 9. Schematic illustrations of melittin bind to (a) HPA- and (b) NHC-supported lipid layer surfaces.

Assuming a steady-state equilibrium between free mellitin (A), the lipid layer (B) and the mellitin-lipid complex (AB)

$$A + B \leftrightharpoons AB \quad (1)$$

the rate equation

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

where the rate constants k_a and k_d represent the complex formation and disruption events at the sensor surface; based on this rate law, equilibrium binding response, R_{eq} may be derived[49,50]

$$R_{eq} = \frac{CK_AR_{max}}{1+nCK_A} \quad (3)$$

where K_A is the binding affinity of mellitin to the surface; C is the concentration of free mellitin in solution, $[A]_t$, effectively constant in this case; R_{max} is the maximum mellitin binding response; and n is a steric interference factor that is used to compensate for steric blocking of additional binding sites by a single analyte molecule.[50] R_{eq} may be obtained from the extrapolation of the binding portion of the adsorption curve shown in the left hand inset of Figures 8 c,d. Using equation (3), the K_A obtained for the HPA chip is (1.05 ± 0.33) × 10^8 M^{-1}. This is much larger than values previously reported on either an HPA or L1 chip (Table 1). Such a high value presumably results from the incomplete lipid layer on the HPA chip surface, with melittin simultaneously forming both a complex with the lipid layer and, through hydrophobic association, to the underlying exposed HPA chip surface. The NHC chip exhibits a K_A of (7.27 ± 0.09) × 10^6 M^{-1}. While this value is somewhat higher than that reported by Shai *et al.*[31] the adsorption/desorption curves are similar in appearance to those reported for melittin binding to a lipid bilayer on an L1 chip. While Shai *et al.*[31] report a K_A value for an HPA chip, they did not publish the adsorption/desorption curves used to obtain these values, so the result is more difficult to compare directly. However, one might note that the R^2 values for the melittin/lipid study performed here on the HPA and NHC chip are 0.72 and 0.92, respectively, illustrating that there is relatively weak correlation between the model and data for the HPA chip as compared to the NHC chip.

Table 1. Affinity data obtained from steady-state analysis.

	HPA chip[a]	NHC chip[a]	HPA chip[b]	L1 chip[b]
K_A (M^{-1})	(1.05 ± 0.33) × 10^8	(7.27 ± 0.09) × 10^6	(1.88 ± 0.09) × 10^4	(4.70 ± 0.30) × 10^5

[a]Data from the present study. [b]Data from Shai *et al.*' study.[31]

Our results show that melittin can bind in the NHC-supported lipid monolayer and the corresponding performance seems to be similar to the L1 chip.[31] The L1 chip exhibits a more widely-spaced and intact bilayer supported by lipophilic groups on its underlying dextran matrix, and previous studies have shown the feasibility of incorporating transmembrane proteins similar to mellitin on L1 chips.[3,4,12] As we have shown, the lower packing density of

the NHC surface led to a formation of a more stable lipid bilayer compared with the densely packed octadecanethiols of the HPA chip. Therefore, the similar mellitin adsorption behavior on the L1 and NHC chips does seem consistent. The lower packing density of the NHC facilitates not only the fast formation of a stable lipid layer, but also the effective intercalation of other species such as peptides or proteins.

CONCLUSIONS

In summary, we have demonstrated that an alkylated NHC SAM on gold surface can provide an efficient platform for fast formation of a stable supported hybrid lipid bilayers. Compared to thiol-based HPA chips, the NHC SAM has the advantages of lower non-specific binding ability, better chemical and significantly superior thermal stability, higher reproducibility, shorter equilibration time, and longer life span in SPR biosensing. The robust performance of the NHC chip that results from the low lability of the C–Au bond allows the usage and storage over a broader range of conditions while maintaining functionality. We also evaluated the performance of a hybrid lipid bilayer formed on the NHC-based platform by assessing the binding between melittin and lipid as compared to HPA and L1 chips. These enhanced properties of the NHC platform offer opportunities to overcome the existing challenges of the thiol-based surfaces. Together, this study demonstrates the feasibility of employing NHC-based platform in SPR biosensing, sheds light on the future design of versatile NHC biosensor surfaces and boosts the practical applications in this direction, thereby enhancing and opening up new opportunities in fundamental biology and healthcare areas.

ASSOCIATED CONTENT

Supporting Information: Additional experimental details, figures, tables are provided in the supporting information available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

* E-mail: jhh@queensu.ca; Phone: +44 (01323) 834467

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