A generalized strategy for immobilizing uniformly oriented membrane proteins at solid interfaces†

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We have developed a method based on self-assembly of thiols on Au substrates to immobilize membrane proteins at interfaces. Using water soluble nitrilotriacetic acid (NTA)-terminated oligo(ethylene glycol) thiols, a histidine-tagged G protein-coupled membrane receptor (GPCR) was captured in a defined orientation with little nonspecific binding.

The fabrication of biomimetic interfaces containing integral membrane proteins (IMPs) is essential for screening of pharmaceutical targets, characterizing the structure and function of IMPs, and development of biosensors.1,2 Key challenges in creating such interfaces include selecting methods for surface immobilization of IMPs at high density with specific orientation, enabling the substrate surface to resist all nonspecific adsorption, and maintaining a fluid lipid environment for the membrane proteins.3

High affinity interactions have been exploited to immobilize recombinant proteins on the surfaces in a defined orientation using tags such as histidine (His), FLAG, and myc.3 In particular, His-tagged proteins are immobilized on nitrilotriacetic acid (NTA)-derivated surfaces in one step, unlike other functionalization strategies of tagged proteins, which require prior immobilization of larger biomolecules such as avidin or antibodies. While NTA-derivated surfaces have been routinely used to capture His-tagged soluble proteins,4 fabrication of IMP-functionalized surfaces using the NTA-His based affinity system has been attempted with only limited success. Friedrich et al. immobilized a His-tagged IMP, cytochrome c oxidase, to a NTA-functionalized surface; however, nonspecific protein adsorption resulted in non-uniform orientation of IMPs on these surfaces.5,6

Surfaces covered with the oligo(ethylene glycol) [OEG] motif have been widely used to attain high resistance to protein adsorption.7,8 However, we observed that the length of ethylene glycol segments within the OEG motif was critical to establishing the extent of resistance to nonspecific adsorption of IMPs. Our protein adsorption studies demonstrate that thiol molecules containing three ethylene glycol segments were able to resist nonspecific binding of detergents,9 an important property since IMPs require detergents and lipids for isolation, i.e. solubilization and stabilization. Prior strategies for membrane protein immobilization have employed OEG-terminated compounds containing long polymethylene chains. However, membrane proteins appear to interact with these long allyl chains, promoting non-specific adsorption, as mentioned earlier.1

On the basis of these results, we synthesized compound 1 (Fig. 1A), terminating with a nitrilotriacetic acid (NTA) moiety, for the selective immobilization of His-tagged proteins,6 and compound 2 (Fig. 1A), a hydroxyl-terminated OEG, to laterally dilute 1 for optimal IMP binding, to sustain resistance to nonspecific adsorption and support the subsequent reconstitution of the lipid bilayer (Fig. 1B).10 The central 1-thiapropylhexa(ethylene glycol) [HS(CH2CH2O(CH2CH2O)6] motif in 1 and 2 is the foundation of a water-based surface functionalization strategy that also has potential use in high-throughput screening platforms including membrane protein microarrays and microfluidic devices using polymers such as PDMS, which are incompatible with organic solvents.11 We carried out a thorough study to correlate surface density of compound 1 and density of specific IMP binding. Precise control of IMP density at interfaces is critical for detailed structural and functional characterization of these proteins using techniques such as neutron scattering and surface plasmon resonance (SPR).12

X-ray photoelectron spectroscopy (XPS) data were used to determine the surface density of compound 1 in the mixed self-assembled monolayers (SAMs) formed at varying solution mole fractions of 1 and 2 from water and under acidic conditions (Fig. 2). Assuming that the adsorption of the two thiols on the
The hypothesis was supported by the 10-fold drop in the adsorption of compound 1 due to a residual negative charge of the NTA groups of the thiol solution (the dashed line in Fig. 2). Under these conditions, the NTA groups of 1 carry a net negative charge, which in turn enhances the repulsive conditions towards further adsorption of 1 during formation of mixed SAMs.

We also employed spectroscopic ellipsometry (SE) to measure the ellipsometric thickness of the SAMs made of 1 and 2. The SE data indicate a nonlinear increase in ellipsometric thickness with increasing surface density of 1. The XPS and SE data demonstrate that the surface density of 1 can be controlled for the fabrication of NTA-functionalized capture surfaces.

We monitored the binding of His-tagged proteins to the NTA-functionalized surfaces prepared by varying the molar ratio of 1 using SPR (Biacore T 100‡) measurements. For protein binding studies, a 44 kDa detergent-solubilized functional G protein-coupled membrane receptor (GPCR), cannabinoid receptor type II with a deca-histidine tag at the C-terminus ([(His)6]CB2)15 and a 32 kDa membrane scaffold protein 1E3D1 ([(His)6]-MSP1E3D1, Sigma-Aldrich) were introduced onto different NTA-functionalized surfaces. The cannabinoid CB2 receptor plays a key role in immune regulation and inflammatory processes, and has become a prime target for therapeutic intervention.16 This highly hydrophobic membrane protein is solubilized in a mixture of the zwitterionic detergent chlamidopropyl dimethylammonio-1 propanesulfonate (CHAPS), the nonionic detergent dodecyl maltoside (DDM), and an anionic cholesteryl ester, cholesteryl hemisuccinate (CHS), and is further stabilized by addition of the strongly binding cannabinoid agonist CP-55,940.17 MSP1E3D1 is a soluble protein which is used to form lipid nanodiscs containing membrane proteins for structural and functional studies.18 MSP1E3D1 is introduced onto the surface in Tris buffer without detergents.

Fig. 3 shows the SPR response of His-tagged CB2 and MSP1E3D1 adsorption on NTA-functionalized surfaces with increasing coverage of 1. SAMs of 2 (θ1, solu = 0; SE thickness ≈ 1.2 nm) were found to be effective in resisting nonspecific adsorption of both proteins, a necessary condition for the development of surfaces that capture membrane proteins. Also apparent from Fig. 3 is that protein binding increases with increasing surface density of 1, reaching saturation of protein adsorption at a mole fraction (θ1, solu) of 0.5 for CB2 and 0.6 for MSP1E3D1. The maximum SPR response of 4000

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Fig. 1  Oligo(ethylene glycol) compounds. (A) Structures of 1 and 2. (B) Schematic illustration of binding of His-tagged detergent-solubilized membrane protein to NTA-functionalized surfaces. NTA-derivatized surfaces were prepared via formation of mixed self-assembled monolayers in water (i) before and (ii) after His-tagged detergent-solubilized membrane protein immobilization.

Fig. 2  Characterization of mixed SAM surfaces of 1 and 2 by XPS. Surface mole fraction of 1 (θ1, surf) as determined by XPS spectra, as a function of mole fraction of 1 in the immersion solution (θ1, solu); solid symbols, 3%aq, acetic acid; open symbols, water [experimental data; Table S1, ESI†]. The lines represent the corresponding fitted curves using eqn (2).

Fig. 3  Protein binding studies on nickel-activated NTA-functionalized surfaces by SPR. SPR responses of protein adsorption – a His-tagged detergent-solubilized cannabinoid receptor (CB2) [unfilled bars] and a His-tagged soluble protein, membrane scaffold protein (MSP1E3D1) [filled bars]7 as a function of mole fraction of 1 (θ1, solu).
Temperature dependent CB2 membrane protein binding studies on NTA-functionalized surfaces. Representative SPR responses of CB2 binding on nickel-activated NTA-functionalized surfaces [prepared from a θNTA = 0.2 solution] before (unfilled bars) and after (filled bars) treatment with imidazole (500 mM) at both 25 °C and 8 °C. Error bars represent the standard error of the mean based on three measurements.

Resonance Units (RU) for CB2 is approximately $3.4 \times 10^{10}$ molecules per mm$^2$. Additionally, this SPR response is comparable to that obtained for the full surface coverage of rhodopsin.\(^{19}\)

To test the specificity of binding of these His-tagged proteins on nickel-activated NTA-functionalized surfaces, the SPR response was measured upon imidazole replacement of captured His-tagged protein. We observed that treatment of the CB2 protein-captured surfaces with 500 mM imidazole at 8 °C resulted in the complete dissociation of the bound CB2, indicating that the membrane protein was specifically bound to the surface (Fig. 4). In contrast, imidazole treatment at ambient temperature (25 °C) resulted in only 70% desorption of the captured CB2. We attribute this binding response to denaturation of CB2 at a higher temperature which resulted in nonspecific adsorption of part of the protein on the surface. This apparent reduction in the specific binding response of His-tagged CB2 protein onto NTA-functionalized surfaces supports our earlier observation of a rapid loss of functional activity of CB2 in detergent solution at ambient temperature.\(^{17}\) By comparison, imidazole treatment of the captured soluble protein MSP1E3D1 at ambient temperature (25 °C) resulted in complete dissociation of the protein from the surface, indicating that MSP1E3D1 was specifically bound to the surface and stable at ambient temperature. The binding of His-tagged MSP1E3D1 could be used for immobilization of membrane protein-containing lipid nanodiscs.\(^{18}\)

We also tested the nonspecific adsorption of these His-tagged proteins on NTA-functionalized surfaces in the absence of nickel. SPR response indicated that nonspecific adsorption was approximately 10% in the absence of nickel as compared to specific binding of His-tagged proteins on nickel-activated NTA-functionalized surfaces. Additionally, we studied the stability of His-tagged proteins on NTA-functionalized surfaces by determining the equilibrium binding constant of the interaction of His tag with NTA. By measuring the SPR binding of MSP1E3D1 to the NTA-functionalized surface ($\theta_{\text{Nas}} = 0.05$) as a function of MSP1E3D1 concentration in solution, we obtained a binding constant of $31.2 \pm 6.9$ nM for this interaction which indicates that His-tagged proteins were stably bound to these surfaces (data are provided in ESI†).

In conclusion, we have demonstrated a facile strategy to fabricate membrane-protein-functionalized surfaces for high-resolution structural and functional studies. We synthesized a water soluble compound 1 for specific capture of His-tagged membrane proteins and an analogous compound 2 for prevention of nonspecific adsorption of these proteins on the surfaces. Moreover, we demonstrated that the membrane protein surface density can be controlled by tuning the surface density of the capture molecule 1 in mixed SAMs. To our knowledge, this is the first successful attempt to immobilize a GPCR protein from a detergent solution on a surface in a defined orientation without a membrane protein carrier\(^{20}\) or use of a tethered large biomolecule.\(^{21}\) We are working towards forming lipid membranes to embed the surface-captured GPCRs, simulating the native lipid environment essential for retaining the structural integrity and functional activity of these proteins.

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Notes and references

\(^{†}\) Certain trade names and company products have been identified in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products are necessarily the best for the purpose.