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Design and characterization of a metal ion–imidazole self-assembled monolayer for reversible immobilization of histidine-tagged peptides†

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We report the design and characterization of a metal ion–imidazole self-assembled monolayer on a gold electrode. The resultant monolayer is well-suited for direct immobilization of histidine and methylene blue-modified peptides.

Since the discovery of nitrilotriacetic acid (NTA), the first 4-coordinate chelating agent, in 1987, immobilized metal affinity chromatography (IMAC) technology has been widely explored and implemented in both chromatographic and non-chromatographic applications.[1–3] The general principle behind the IMAC technology is the use of a metal ion to coordinate between pyrrole amine ligands and a surface immobilized ligand such as NTA. One of the most valuable applications of IMAC technology to date has been protein purification. Recombinant proteins are often engineered to express hexa-histidine tags, thereby allowing them to be easily separated from cellular debris. Potential applications of IMAC technology are not limited to chromatographic separations; more recently, surface-immobilized NTA has been used in the fabrication of biosensors. In a previous study, a NTA-terminated monolayer was used to immobilize Photosystem II (PS II) for detection of herbicides.[4]

In addition to NTA, various histidine-binding ligands have been identified and subsequently characterized for their ability to coordinate with histidine-tagged (His-tagged) proteins. Among them, one of the less-studied ligands is imidazole (Im), even though it is commonly employed as the displacement ligand in nickel-NTA columns.[5,6] This ligand's capability in capturing His-tagged proteins or peptides onto a solid support has not been investigated. Nonetheless, thiolated Im has previously been used in the immobilization of redox proteins through their accessible metal centre.[5,6] These studies suggest that direct attachment of redox proteins on an Im-containing self-assembled monolayer (SAM) can lead to an enhancement in the electron transport property. In spite of these advancements, thiolated Im has not been used in the fabrication of electrochemical biosensors such as the electrochemical peptide-based (E-PB) sensor.[7,8]

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The development of E-PB sensors is the first step towards establishing a sensing platform that can potentially rival the current gold standard in antibody detection—the ELISA test. To date, the most straightforward method to fabricate an E-PB sensor has been the conventional "two-step" approach, which involves direct adsorption of thiolated peptide probes onto the gold electrode surface.[7] Despite being a valuable probe immobilization strategy, the "two-step" approach lacks general surface modification versatility. "Click" chemistry, a more versatile sensor fabrication approach, has recently been developed; however, it is considered as a permanent surface conjugation method.[9,10] Unlike the "two-step" or "click" chemistry approach, the metal–ligand peptide immobilization approach offers a significant advantage of versatility in sensor fabrication.

In this study, we investigated the feasibility of utilizing the metal ion–Im platform for immobilization of a surrogate E-PB probe—a hexahistidine-modified methylene blue (His_6-MB; Fig. SI1, ESI†). While the exact binding mechanism has not been elucidated, it is commonly believed that the coordination between a His ligand and a metal ion is dependent on the surface metal–ligand interaction.[11,12] Previous studies also suggest that once the metal ion is coordinated to the surface-immobilized ligand, the most stable conformation between the metal ion and the His-tag is achieved through a 2-site coordination.[11–14] The use of metal–ligand interactions in the fabrication of E-PB sensors is advantageous since it is not a covalent linkage; the semi-permanent nature of this peptide immobilization strategy allows a single monolayer to be reused for subsequent immobilization of a different peptide probe. In addition, the Im ligand, in comparison to the NTA ligand, has a smaller, more compact head group, enabling the formation of a more tightly packed monolayer, which could potentially enhance monolayer stability.[15] We thus believe our results could be of significance not only in E-PB sensor fabrication, but also in the design and fabrication of other biosensors that require immobilization of His-tagged peptides and proteins.

In brief, the monolayer used in this study was formed using a "two-step" approach, in which 1-(11-mercaptoundecyl)imidazole (C11-Im) was first adsorbed onto the gold disk electrode, followed by subsequent adsorption of the passivating diluent, 8-mercapto-1-octanol (C8-OH) (Fig. 1). The mixed monolayer was then exposed to metal ions for direct coordination with the surface-immobilized Im ligands. While various cations

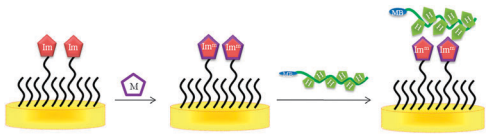
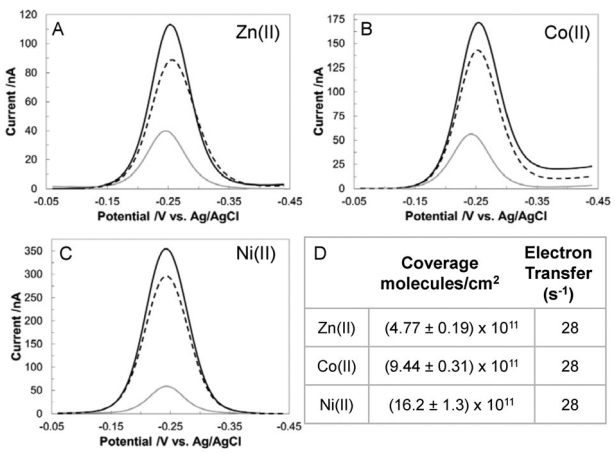


Fig. 1 Schematic showing the steps used in His_6-MB probe immobilization which includes a metal ion chelation step. The SAM is composed of C11-Im and C8-OH. Divalent cations used in this study include Zn(II), Co(II) and Ni(II) (also see Fig. SI2, ESI†).

have been known to coordinate with Im ligands, we decided to study the three most commonly used metal ions in IMAC applications, namely zinc (Zn(II)), cobalt (Co(II)), and nickel (Ni(II)). The last step of the electrode modification protocol involved the introduction of His_6-MB into the metal-containing monolayer. Other fabrication protocols have also been attempted; however, the resultant MB signal is relatively unstable (Fig. SI3, ESI†). Although the exact reason behind the effect of the SAM formation protocol on the stability of the coordinated His_6-MB probe is not well understood at this stage, it is conceivable to assume that two Im ligands are required to bind to one metal ion in order to maintain a stable configuration for efficient binding of His_6-MB.[11–14]

Alternating current voltammetry (ACV) was used to characterize the three His_6-MB modified SAMs. Fig. 2 shows the AC voltammograms of the three SAMs collected in a PBS solution after monolayer equilibration, ligand-induced His_6-MB displacement, and subsequent His_6-MB replacement. All three SAMs show a sharp, well-defined AC voltammetric peak consistent with the formal potential of the MB redox label. The MB peak current obtainable at the three different SAMs is relatively different, with Ni(II) SAM showing the largest peak current. Co(II) and Zn(II) SAMs display a similar MB current, albeit significantly lower than that observed with Ni(II) SAM. These results are unexpected as Zn(II) has been reported to have the strongest coordination to both Im and His.[16] However, we found that the binding kinetics of His_6-MB to Zn(II) SAM is slow, especially when compared to the other metals used in this



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	Coverage molecules/cm^2	Electron Transfer (s^{-1})
Zn(II)	(4.77 ± 0.19) x 10^{11}	28
Co(II)	(9.44 ± 0.31) x 10^{11}	28
Ni(II)	(16.2 ± 1.3) x 10^{11}	28

Fig. 2 AC voltammograms of the three Im-containing SAMs with (A) Zn(II), (B) Co(II), and (C) Ni(II) as the chelating metal. Shown are scans collected after SAM equilibration (solid black line), first His_6-MB displacement using Im (grey line), and first His_6-MB replacement (dashed black line). (D) His_6-MB probe coverages and electron transfer rates obtained for the three SAMs. The probe coverages shown are averages obtained from three different monolayers/electrodes.

study.[16] Furthermore, increasing the incubation time in the metal solution or His_6-MB solution does not result in a significant increase in His_6-MB probe coverage.[17] Despite our effort to optimize this system, Zn(II) SAM does not appear to be well-suited for His-tagged peptide immobilization. Unlike Zn(II) SAM, Co(II) SAM exhibits slightly faster binding kinetics towards His_6-MB. Probe saturation can be readily achieved at twice the incubation time required for Ni(II) SAM to reach probe saturation (Fig. SI4, ESI†). Among the three SAMs used in this study, Ni(II) SAM displays the highest His_6-MB probe coverage (1.62×10^{12} molecules cm^{-2}) despite the shortest probe incubation time, suggesting that it is the most effective metal ion to be used in the immobilization of His-tagged peptides in E-PB sensor fabrication.

Since metal–ligand interactions are not permanent, His_6-MB could essentially be displaced and subsequently replaced by a different His-tagged peptide. Thus, it is advantageous to develop an efficient probe displacement and replacement strategy to be used with this new peptide-immobilization platform (Fig. SI5, ESI†). As shown in Fig. 2, displacement of His_6-MB was achieved for all three SAMs used in this study by incubating the electrode in 250 mM Im (pH 9.2) for 20–30 min. Zn(II) SAM shows a loss of 65% of the His_6-MB signal, whereas Co(II) and Ni(II) SAMs display a loss of 57% and 90%, respectively. It is worth mentioning that the remaining current observed for the three SAMs is similar in magnitude to the MB peak originated from non-specific adsorption (Fig. SI6, ESI†), suggesting that probe displacement by Im is efficient for all three systems. Unfortunately, the non-specifically adsorbed His_6-MB cannot be easily removed by a competing ligand such as Im. The relatively smaller % signal change observed for Zn(II) and Co(II) SAMs is due to the low initial MB current. We chose to use Im in the displacement assay as it is the most typical eluent used in IMAC technology for His-tagged protein separation.[1–3] While it is possible to use concentrations lower than 250 mM, we found that the displacement is not as efficient and the results are not as reproducible (Fig. SI7, ESI†).

In addition to being able to withstand the displacement step, the three SAMs were adequately stable to undergo a replacement step. Unlike the displacement step, the replacement step is slightly more complex (Fig. SI2, ESI†). Specifically, the post-displacement SAMs require replenishment of the lost metal ions prior to the actual replacement of the His_6-MB probes. This metal ion replenishment step serves several purposes, including the removal of Im bound to the surface, replacement of metal ions into the sites where they were displaced, and filling in new empty sites that became available after monolayer rearrangement. Additionally, the metal ions could also assist in removing non-specifically adsorbed His_6-MB that could not be removed by the displacement step. Overall, the metal ion replenishment step appears to improve monolayer homogeneity, which is necessary for optimal His_6-MB coordination during the replacement step (Fig. SI8, ESI†). Despite the slightly more cumbersome protocol, the MB signal observed post-replacement is relatively similar to the original MB signal (Fig. 2), suggesting that this protein immobilization platform is regenerable and reusable.

The ability to reuse a single sensor multiple times is a valuable attribute of this class of folding-based electrochemical

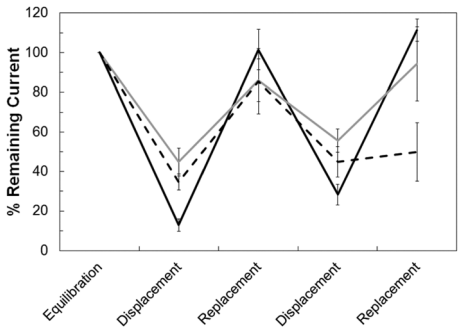

Fig. 3 Shown are the displacement–replacement cycles observed with the three SAMs used in this study. Ni(II) SAM (solid black line) displays highest probe displacement and replacement efficiency in both cycles when compared to Zn(II) (dashed black line) and Co(II) (grey line) SAMs. The data are averages of three different SAMs for each metal ion system.

biosensors. Although the use of this platform will not result in a sensor that is reusable based on the conventional definition, the ability to reuse the same monolayer multiple times for immobilization of different peptide-based biosensing elements is equally advantageous. In particular, this application will be of significance if implemented on an integrated microfluidic device. Therefore, we explored the feasibility of using one SAM repeatedly, with the goal of determining the limit of reusability for all three SAMs. As shown in Fig. 3, Zn(II) SAM can only go through one displacement and one replacement cycle. The second replacement cycle is inherently inefficient. Co(II) SAM appears to be reasonably efficient in both the displacement and replacement steps for both cycles; however, the standard deviations are quite large, suggesting the presence of variations among the three Co(II) SAMs used to collect these data. Ni(II) SAM exhibits the most reproducible responses and was thus used in the remaining part of this study.

While Im has shown to be an effective displacement reagent, there are other ligands that could be equally effective. Fig. 4 shows a direct comparison of the efficacy of probe displacement by Im and two other ligands, L-histidine and histamine. Since all three molecules have an Im functional group and are biological molecules that have been used in IMAC columns, they should be equally effective as displacement ligands. However, we found that if used at pH 8.6, L-histidine is

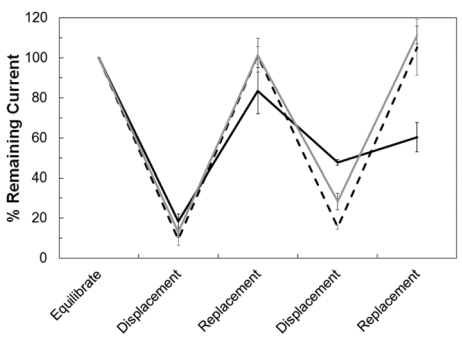

Fig. 4 Shown are the displacement–replacement cycles observed with Ni(II) SAMs. The signal suppression observed in the first displacement is ~82% for L-histidine (solid black line), ~91% for histamine (grey line), and ~87% for Im (dashed black line). The signal suppression obtained in the second displacement is ~53% for L-histidine, ~85% for histamine, and ~70% for Im.

incapable of displacing His_6-MB in the second cycle, which could contribute to the inefficient replacement step. Further investigation revealed that if used at pH 7.4, L-histidine is an equally efficient and effective displacement reagent when compared to Im and histamine (Fig. SI9, ESI†).[18] The reason behind this behaviour is unclear and is currently under investigation in our lab. Unlike L-histidine, both histamine (pH 11.4) and Im (pH 9.2) are effective in displacing His_6-MB without a pH adjustment. Both ligands could also be used at pH 7.4. Thus, depending on the chosen pH, all three displacement ligands are equally effective, but since Im is more cost-effective than the other two molecules, it will be the displacement ligand of choice in our future E-PB study.

In summary, we show for the first time the use of C11-Im for reversible immobilization of His-tagged peptides. All three metal ions are capable of coordinating to the surface-immobilized Im terminal group, with Ni(II) SAM exhibiting optimal His_6-MB probe capture capacity. It is worth noting that in addition to His_6-MB, we used another MB-modified peptide to demonstrate that this platform can be used with a longer peptide probe (Fig. SI10, ESI†). Despite the difference in the length of the peptides, the captured probes can be effectively displaced by competing ligands, and more importantly, the same probes can be readily replaced. The semi-permanent nature of this probe immobilization strategy can be exploited in the serial fabrication of multiple E-PB sensors on the same monolayer. This strategy is currently being investigated in our lab, with the goal of fabricating a series of E-PB sensors for detection of peanut allergen antibodies.

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