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A reagentless electrochemical biosensor based on a protein scaffold†

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Apo-myoglobin, labeled with the environmentally sensitive redox probe $Ru^{II}(NH_3)_4$(1,10-phenanthroline-5-maleimide)$^{2+}$, was immobilized onto gold electrodes modified with 11-mercaptoundecanoic acid and subsequently labeled with biotin; avidin binding to the immobilized biotin was specifically and quantitatively detected by a change in cyclic voltammetry of the co-immobilized probe.

Reagentless transduction of molecular recognition at electrode surfaces has been the subject of several research efforts[1–5] with the goal of developing electrochemical biosensors[6–8] not limited by manipulation of reagents (such as enzyme-labeled secondary antibodies). Here we introduce an approach to reagentless biosensing in which an environment sensitive redox probe and a selective binding unit (in this case, a ligand to a protein target) are co-conjugated to an electrode-immobilized protein (scaffold). Binding of target protein to the ligand is reported as a change in voltammetry of the probe. This approach utilizes the diversity of functional groups on protein surfaces for conjugation and the blocking property of electrode-immobilized proteins with respect to non-specific protein adsorption. The proof of concept demonstrated here utilizes biotin as the binding unit and avidin as the target. Because a wide range of candidate binding units exist (such as enzyme cofactors) or can be synthesized in libraries (such as oligosaccharides, peptides and aptamers) and selected against multiple protein targets, this approach may eventually enable reagentless detection of a wide range of proteins and prove a useful tool in proteomics.

Genetically engineered mutant sperm whale apo-myoglobin, apo-HTC64Mb, with a hexa-histidine tag (HT) at the C-terminus (for purification) and containing a single sulfhydryl function (contributed by cysteine at position 64) was used as the protein scaffold.† To ensure that only the heme-less or apo form of HTC64Mb was used in the experiments, recombinant C64Mb was purified from *E. coli* cells under denaturing conditions and subsequently allowed to refold in the absence of denaturant.

Conjugation of $Ru^{II}(NH_3)_4$(1,10-phenanthroline-5-maleimide)$^{2+}$, a previously reported,[6] sulfhydryl-reactive, environmentally sensitive redox probe, to cysteine 64 of apo-HTC64Mb resulted in the redox labeled protein, RuMb. The absence of any residual heme cofactor and presence of the covalently attached Ru-complex were verified using UV absorbance spectroscopy.[6,9] Cleaned gold wire electrodes[10] modified with 11-mercaptoundecanoic acid (11-MUDA) were used for either electrostatic immobilization or covalent immobilization of RuMb by covalent coupling using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride, (EDC, Sigma) of RuMb.[11,12] Following immobilization, RuMb was reacted with lysine specific NHS-LC-LC-biotin (Pierce). Immersion of a resulting biotin-labeled RuMb (biotin-RuMb) modified electrode into buffer and stepwise addition of avidin between ~ 5 nM to 500 nM resulted in a stepwise decrease in cyclic voltammetric peak current of the redox probe (Fig. 1). No change is observed in double-layer capacitance charging current upon avidin binding as expected owing to the thickness of the monolayer and scaffold layer.[13]

Table 1 compares biotin-RuMb immobilized using both methods with and without bound avidin (500 nM) with respect to formal potential ($E°$), average of background subtracted anodic and cathodic peak current (I_p), apparent electrode coverage measured by peak current integration, (Γ) and actual electrode coverage inferred by thickness of the protein layer(s) measured by *ex situ* ellipsometry (T).[15] From Table 1 the following observations are made. (1) Both immobilization methods result in similar protein layer thicknesses consistent with a monolayer of myoglobin bound to each electrode and subsequent binding of a monolayer of avidin.[16–18] (2) The apparent electrode coverage of EDC-coupled biotin-RuMb is ~ 0.2 times that of electrostatically absorbed biotin-RuMb. This discrepancy between observations one and two is consistent with results reported by Collinson *et al.*[12] comparing covalently *vs.* electrostatically immobilized cytochrome *c* on carboxylic acid terminated monolayers on gold electrodes. Their results

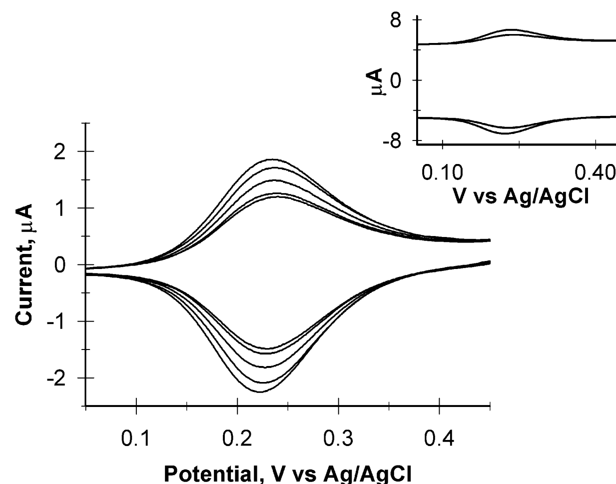

Fig. 1 Cyclic voltammetry[14] of biotin-RuMb EDC coupled to a 11-mercaptoundecanoic acid-modified gold electrode titrated with avidin, 2 V s^{-1}, background subtracted. **Inset**. Cyclic voltammetry without background subtraction with and without 500 nM avidin.

Table 1 Properties of biotin-RuMb immobilized on a gold modified electrode[a]

	E^{0b}/mV	$I_p{}^c$/μA	Γ/pmol cm^{-2}	T^dÅ
Electrostatic	218	11.6 ± 1.3	6.8 ± 0.3	42 ± 5
+ 500 nM avidin	228	8.7 ± 0.9	5.1 ± 0.4	113 ± 7
EDC coupled	231	2.3 ± 0.7	1.3 ± 0.3	36 ± 12
+ 500 nM avidin	238	1.4 ± 0.5	0.8 ± 0.2	97 ± 4

[a] Average of 2–3 measurements. [b] Average of anodic and cathodic peak potentials *vs.* Ag/AgCl. [c] Average of background subtracted anodic and cathodic peak currents. [d] Average of 5 measurements from 2 samples each.

† Electronic supplementary information (ESI) available: details regarding protein engineering and purification. See http://www.rsc.org/suppdata/cc/b2/b209452e/

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demonstrated that heterogeneous electron-transfer is sensitive to the orientation of the redox cofactor (heme) of cytochrome *c* with respect to the underlying electrode. As in that case, it is hypothesized here that covalent immobilization of RuMb results in random orientation of redox probes with respect to the underlying electrode. In contrast, electrostatic adsorption may favor orientations of RuMb in which positively charged redox probes are directed, on average, toward the negatively charged monolayer surface, and thus the underlying electrode, thereby enhancing electron-transfer kinetics.[19] (3) For both immobilization methods, there is a shift in formal potential (modest) and a decrease in peak current (pronounced) after addition of avidin. These results are consistent with those reported by Yan *et al.*, describing binding of anti-biotin to electrodes modified with self-assembled monolayers containing biotin, viologen and polyethylene glycol terminated alkylthiolates.[1] As in that case, it is hypothesized here that the observed voltammetric changes reflect changes in the immediate environment of the redox-probe concomitant with target binding. We do not yet understand the mechanism resulting in the observed voltammetric changes. Its elucidation is a goal of on-going investigations. (4) The relative reduction in peak current upon avidin binding is greater for EDC-coupled biotin-RuMb (38%) than for electrostatically immobilized biotin-RuMb (25%). This result is consistent with immobilization dependency on orientation of biotin-RuMb. Binding of avidin to the outer surface of biotin-RuMb should affect to a lesser extent the microenvironment of redox probes that are directed, on average, toward the underlying electrode surface.

The peak current of modified electrodes with biotin-RuMb, immobilized by either method, is responsive to incremental additions of avidin (Fig. 2). The detection limit of avidin demonstrated here is ~15 pmol in a 3 mL sample volume for electrodes with area = 1.2 ± 0.15 cm^2 and a coverage of 6.8 ± 0.4 pmol cm^{-2} of immobilized biotin-RuMb.

Specificity is demonstrated by the observation that peak current minimally changes (<5%) under the following conditions. (1) Immobilized RuMb not labeled with biotin is exposed to otherwise saturating avidin (125 nM). (2) Immobilized biotin-RuMb is exposed to excess (1500 nM) bovine serum albumin (BSA) in place of avidin. (3) Immobilized biotin-RuMb is exposed to free biotin (1 μM biotin) prior to exposure to avidin.[20] Furthermore, addition of excess BSA does not interfere with detection of subsequently added avidin. To rule out possible electrostatic interactions between the protein target and monolayer contributing to current decrease, detection of avidin was shown to work equally well in higher ionic strength phosphate buffered saline (PBS).[21] In addition, NeutrAvidin (Pierce), a variant of avidin with a neutral pI, generated a nearly identical dose response to that of avidin (pI = 10).

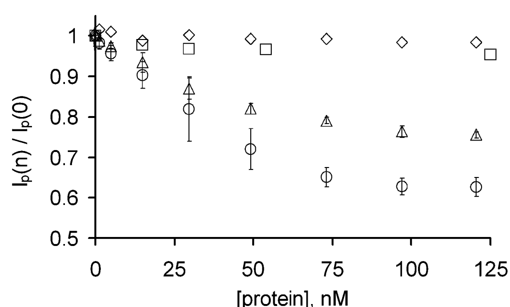

Fig. 2 Peak current normalised to initial peak current of immobilized RuMb *vs.* concentration of added protein. (◇) EDC-coupled biotin-RuMb with BSA; (□) EDC-coupled RuMb (non-biotinylated) with avidin; (Δ) electrostatically adsorbed biotin-RuMB with avidin; (○) EDC-coupled biotin-RuMB with avidin.

Detection of avidin shown here is intended as a proof-of-concept demonstration of scaffold-based electrochemical reagentless detection. The key attributes of using electrode-immobilized proteins as biosensor scaffolds include diversity of candidate ligands, diversity of chemical moieties for covalent attachment of probes and ligands, potential to adjust sensitivity by control of probe–ligand orientation through site-directed mutagenesis, and blocking of non-specific adsorption of non-target proteins.

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