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Peptide Nanowires for Coordination and Signal Transduction of Peroxidase Biosensors to Carbon Nanotube Electrode Arrays

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Abstract

A strategy of metallizing peptides to serve as conduits of electronic signals that bridge between a redox enzyme and a carbon-nanotube electrode, has been utilized with enhanced results. In conjunction, a protocol to link the biological elements to the tips of carbon nanotubes has been developed to optimize contact and geometry between the redox enzyme and the carbon nanotube electrode array. A peptide nanowire of 33 amino acids, comprised of a leucine zipper motif, was mutated to bind divalent metals, conferring conductivity into the peptide. Reaction between a thiolate of the peptide with the sulfenic acid of the NADH peroxidase enzyme formed a peptide-enzyme complex that are fully primed to transduce electrons out of the enzyme active site to an electrode. Scanning electron microscopy shows immobilization and linking of the assembly specifically to the tips of carbon nanotube electrodes, as designed. Isothermal titration calorimetry and mass spectrometry indicate a binding stoichiometry of at least three metals bound per peptide strand. Overall, these results highlight the gain that can be achieved when the signal transducing units of a biosensor are aligned through directed peptide chemistry.

Keywords

peptide nanowire; carbon nanotube electrode; NADH peroxidase; gold nanoparticle; metallized peptide; nanoarrays

1. Introduction

The application of biological macromolecules as detectors provides a means of monitoring biorecognition events and interactions on nanoplatforms. Coupled to the ability to precisely produce conductive elements on the nanoscale, biosensing offers unprecedented avenues for screening and detection at increasing sensitivities. We have integrated the precise three-dimensional structural information obtained from X-ray crystal analysis with nanotechnology platforms. This permits the formation of modular sensors, allowing optimization of the geometrical parameters comprising the various components of a

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biosensor. These nanobiosensors generally consist of a biological molecule, a linker or mediator, and nanoelectrode arrays. The various components can be equated with the electronic elements of a physical sensor as every component has to transduce the signal generated at the source (i.e. biomolecule) to the detector (i.e. electrode). As biomolecules are integral sensing units, fundamental kinetic parameters of interaction between individual molecules and their surrounding dictate the rates of signal transduction, as in native biological systems. Consequently, as in enzymes, rate improvements can occur from proximity and geometric effects, with potential enhancements of 10^2 to 10^3 at each junction. This geometric benefit is intrinsically available in carbon nanotubes and can be exploited if individual nanotubes can be accessed physically and electrically, as is the case and demonstrated in this work. This benefit is multiplied when precise, atomically resolved coordinates of biological structures are integrated rationally on the nanoelectrode platform, as described in this report.

Although there is much activity using nucleic acids in biosensors, due to their inherent native charge, sequence recognition properties, and stability (Drummond, et al., 2003; Gao, et al., 2007; Ito et al., 2007), applications using peptides and proteins are appealing alternatives. However, using proteins in this manner are complicated by a multitude of factors including preserving the functional state and folding of the macromolecule and harnessing signals generated at buried active sites so they can be directed to electrode surfaces (Armstrong, 2005; Willner et al., 2006). In this report, we describe the application of a highly stable helical peptide that can bind multiple metals per strand. These peptides are facilely functionalized for reaction to specific protein sites such that one termini of the peptide reacts with the biomolecular source (i.e. enzyme) and the other end with the nanoelectrode tip, with the metallized peptide acting as a conductive bridge between the two components. This allows for the modular production of biosensors, permitting the protein and electrode components to be readily swapped and replaced. Modularity in design allows facile production of biosensors to detect a large array of ligands.

As a strategy to optimize the distance and conformation of each component of the biosensor, comprised of the redox enzyme NADH peroxidase (Npx), peptide linker (the complex of the enzyme with peptide hereon referred to as “biomolecular complex”), and electrode, the peptide serves multiple purposes. Its stable helical structure serves as a spacer between the protein and the electrode surface, helping to prevent surface denaturation. Functionalization of the peptide allows for specific linkage to the protein and electrode, giving rise to a more homogenous distribution of the assembly on the electrode surface. The peptide’s structural integrity and elongated conformation permits its penetration into the active site of the enzyme with little perturbation to the enzyme’s structure and provides a means of directing the electrons generated at the enzyme center to the electrode.

Confirmation of specific placement of the biomolecular complex to the tips of carbon nanotube electrodes was obtained through reaction of gold-nanoparticle (Au-NP) labeled bioassembly using a stoichiometric labeling approach, described here. First, the peptide was linked to the protein, followed by the labeling of the complex with a monofunctionalized gold nanoparticle of 1.4 nm. The peptide was then metallized with cobalt. An electrode-less deposition approach to enhance the gold nanoparticle’s size to aid in visualization via scanning electron microscopy (SEM) was done. This permitted controlled growth of the nanoparticle size as a phasing reagent while allowing for stoichiometric linkage of nanoparticles to the bioassembly. Once the peptide-protein reaction was completed, the whole assembly was purified from non-reacted material through chromatography approaches.

The potential advantages of utilizing atomic resolution structures and coordination with nanotechnology applications were discussed in some details earlier (Yeh et al., 2005). Measurements of rates of reactions achieved in this system suggested that the transducing units of the enzyme were well aligned for electron transfer. Sensitivity of this system to reactive oxygen species generated in-situ was recently demonstrated (Xia et al, 2006). Earlier, the fabrication and use of CNT electrode arrays as nanobiosensor components were demonstrated in a biosensor using glucose oxidase as the enzyme component, directly anchored by the COOH group at the nanotube tip (Withey et al, 2006). Additionally, the fabrication of biosensors using gold electrodes integrating the same enzyme that is used for this study, Npx, as the biological component was reported with enhanced electron transfer rates (Yeh et al, 2005).

In the current study, we report the specific linkage of a bioassembly to CNT electrode arrays using metallized peptides as conduits of the electronic signal. Solution conditions to enhance reactivity between peptide-enzyme and the subsequent bioassembly were developed. These results highlight the unique electrical and reaction properties of CNT and the potential for coordinated linkage of biological components using metallized peptides to produce highly sensitive biological nanosensors.

2. Materials and Methods

N-hydroxysuccinimide (NHS), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC), β -nicotinamide adenine dinucleotide reduced form (NADH) and tris (2-carboxyethyl) phosphine (TCEP) were purchased from Sigma. Hydrogen peroxide (30%) was from Mallinckrodt. Peptides were made by Bio-Synthesis, Inc. Monofunctionalized gold nanoparticle (AuNP), mono-*sulfo*-NHS-nanogold, was from Nanoprobes. UV-visible absorption measurements were carried out on a SPECTRAmax[®] PLUS³⁸⁴ Spectrophotometer from Molecular Devices. MALDI-TOF-MS spectra were obtained on a Voyager DE-PRO Biospectrometry Workstation. All scanning electron microscope images were taken with a JEOL-1200 SEM.

2.1. Enzyme expression, purification, activity assay

The NADH peroxidase (Npx) of *S. faecalis* was expressed, and tested for enzyme activity as described (Poole and Claiborne, 1986, Parsonage, et al., 1995). The enzyme was purified with a HiTrap Q column from GE Healthcare, eluted with 20 mM Tris/HCl pH 7.5 and 0–1 M NaCl in the presence of 1 mM TCEP. The enzyme was concentrated to >10 mg/mL with an intensely yellow color from the flavin cofactor and stored at –80 °C. In a standard assay condition, the solution mixture consisted of 0.3 mM EDTA, 0.16 mM NADH, and 1.3 mM H₂O₂ in 0.1 M potassium acetate buffer, pH 5.4. NADH was added just prior to assay to avoid nonenzymatic loss. The assay was started by adding 1 μ g/ml Npx, and monitored by the decrease in 340 nm absorbance of NADH.

2.2. Peptides

Sequences and electrochemical measurements on a number of peptides, their binding characteristics to divalent metals and electron-transfer properties were characterized and reported earlier (Yeh et al., 2005). Briefly, the leucine-zipper region of a transcriptional factor, GCN4, was synthesized and this comprised of 33 amino acids in length. Mutations of residues to histidines were made at *i*, *i*+4 positions to bind metals at the outer faces of peptides and a previously reported multiple histidine peptide (MHP) (Yeh et al., 2005) was used for the majority of the studies reported here. A thioester moiety at the carboxyl-termini of the peptide or a sulfhydryl group from a cysteine was designed to react with the active site thiolate of Npx to form a covalent linkage.

2.3 Metallization of Peptide and Mass Spectrometry

The binding of metal ions to the MHP was studied using isothermal titration calorimetry (ITC), as reported earlier (Yeh et al., 2005) and mass spectrometry (MS) methods, in this study. The MS samples were made by mixing MHP peptide with CoCl_2 in a ratio of 2 mole of Co^{2+} per mole of histidine, accounting for the multiple histidines in each chain. Reaction conditions were 10 mM Tris-HCl at pH 7.0, 8.0, 8.5 and 9.0 respectively. Excess Co^{2+} were removed by exchanging the buffer to 10 mM Tris-HCl at the respective pH with YM-3 concentrators (Millipore). The products were subsequently mixed with a matrix of saturated α -cyano-4-hydroxycinnamic acid (CHCA) in 0.3% trifluoroacetic acid and 50% acetonitrile in water in preparation of MALDI -TOF-MS spectroscopy.

2.4 Conjugation of Peptide to Enzyme and Metallization

Enhanced reaction of the enzyme with peptide was achieved by first slightly unfolding the enzyme in guanidine hydrochloride followed by oxidation to form the linkage to Npx. To do this, 50 μg (1 nmol) of Npx was dissolved in 0.2 ml of Tris-HCl, pH 8.0 with 1.0 M guanidine. Hydrogen peroxide (1 nmol) was added and incubated at 0 °C for 5 to 30 min. Peptide MHP (5 nmol) was then added and incubated at 4 °C overnight. Hydrogen peroxide was added to Npx stoichiometrically, converting the Npx Cys-thiol group to Cys-sulfenic acid which can easily react with peptide Cys-thiol group to form disulfide bond. The Npx-MHP complex was purified with a HiTrap Q High Performance column, eluting with 20 mM sodium phosphate buffer pH 7.4 with 0.15–1.0 M NaCl. Complex of Npx-MHP with >95% purity was eluted at around 0.3 M NaCl. The peptide was then metallized with Co^{2+} by mixing Npx-MHP with CoCl_2 .

2.5. Labeling of Bioassembly

Npx bioassembly was labeled with 1.4 nm AuNP, monofunctionalized with a sulfo-1,4-succinimido group (Nanoprobe) for reaction to primary amines. In a representative procedure, 6 mol of AuNP was dissolved in 200 μl dH_2O immediately before use, then added to the Npx bioassembly, containing 60 μg of Npx-peptide in 100 μl PBS buffer (20 mM sodium phosphate and 0.15 M sodium chloride, pH 7.4). The mixture was incubated at 4 °C overnight. Excess AuNP was removed by concentrating with YM-30 concentrator, washed with PBS buffer, and then concentrated again. This efficiently removed unbound AuNP through size partition, since the bioassembly is ~64 kD (Npx enzyme 49 kD, AuNP 15 kD), readily retained from passing through the membrane while free nanoparticles easily permeate through. The AuNP labeled Npx bioassembly was further purified by gel-filtration to isolate the fully-labeled bioassembly.

2.6. Reaction of Bioassembly to CNT

The CNT electrodes were subjected to reactive ion etching. This procedure simultaneously opens the tips while producing a high density of carboxylic acid groups at the tips for subsequent peptide linkage reactions. Npx linking to the CNT tips was accomplished with well known amine coupling procedures (Withey et al, 2006) to the CNTs free carboxyl groups. The carboxylate groups on the CNT electrode tips were activated by pre-treating with a freshly made EDC -NHS mixture at room temperature for 1 h, which contains 0.4 M EDC/HCl and 0.1 M NHS in water (pH 6 to 7). The pre-treated electrode was rinsed thoroughly with PBS buffer and de-ionized water and then treated with Npx assembly in PBS buffer, pH 7.4 at 4 °C overnight.

2.7. Imaging of CNT-Bioassembly

Scanning Electron Microscopy (SEM) was used to image gold nanoparticle labeled Npx-bioassembly conjugated at the tips of highly ordered carbon nanotubes (CNT) arrays. The

highly ordered CNT arrays were beneficial to forming evenly distributed bioassembly, which was confirmed through the SEM imaging. They are fabricated through growth of multiwalled nanotubes (MWNTs) within an aluminum oxide nanopore template (Li et al., 1999, Papadopoulos et al., 2002). The template defines the geometric features of the CNT array, with the three dimensional structure of the sensor elucidated in Figure 1. For the applications described here, the CNTs are 50 nm in diameter, have walls of 3 nm thickness, and exhibit an exposed length of 60 nm, a total length of 10 μ m, and a center-to-center spacing of 100 nm between adjacent tubes as verified Figure 1 (All scanning electron microscope (SEM) images were taken with a LEO 1530 SEM).

2.8. Electrochemical measurements

Electrochemical experiments were performed on an Epsilon Electrochemical Workstation from BASi. The modified CNT electrodes (i.e. working electrodes) and controls, were cycled in 0.1 M potassium acetate buffer, pH 5.4. The reference electrode was a Ag/AgCl electrode and a platinum wire electrode was used as the auxiliary electrode.

3. Results and Discussion

CNTs are of great utility, particularly when they are highly ordered and vertically aligned as in the arrays used in the work described here. These geometrical parameters are advantageous for bio-functionalization and bio-sensing applications (Shim et al., 2002; Sotiropoulou and Chainiotakis, 2003; Tsang et al., 1994; Wang et al., 2003a ; Wang et al., 2003b; Yang et al., 2007) as they exhibit high conductivity and can be modified with site specificity (Withy et al, 2006, Taft et al., 2004) as their closed sidewalls and open ends exhibit inherently different physical and chemical properties (Saito et al., 1998, Dresselhaus et al., 2001). Linkage between CNTs and enzymes via metallized peptides provides a mean of coordinating formation between the various components, particularly accessing and aligning to the enzyme's active site. The Npx enzyme exists in the thiolate state under reducing conditions of the reaction to form the bioassembly. The activity (Poole and Claiborne, 1986, Parsonage, et al., 1995) of the enzyme was determined prior to assembly formation to ascertain the reduced state of the cysteine and competency for disulfide bond formation under all conditions, including in the presence of GnHCl and peptide (Table 1).

To form the linkage between enzyme and peptide, oxidation to form a disulfide (or a thioester bond, depending on the functional group synthesized) is catalyzed by hydrogen peroxide under mild conditions. If the concentration of hydrogen peroxide is in excess, the peroxide could potentially oxidize the Npx Cys-sulfenic acid (Cys-SOH, secondary redox center) further to Cys-sulfinic (Cys -SO₂H) and/or -sulfonic acid (Cys -SO₃H), oxidation states that are unable to react with peptide's Cys-thiol group (Yeh and Claiborne, 2002; Claiborne et al., 2001; Claiborne et al., 1999). Thus, the amount of hydrogen peroxide was controlled and limited to a quantitative ratio of Npx in order to maximize Npx-peptide formation.

For labeling, colloidal conjugation of metals and semiconductors to biological molecules packed into specific geometrical arrangements is the subject of intense scientific investigation (Xiao et al., 2002; Xu et al., 2005; Xu et al., 2007). Bioconjugated colloidal structures have been used for a wide variety of higher order structures including multi-particle complexes (Alivisatos et al., 1996; Hazarika et al., 2007; Yao et al., 2007), DNA scaffolds (Xiao et al., 2002; Helfrich et al., 2005; Yao et al., 2007), nanotube hybrids (Taft et al., 2004) and immunological identification platforms (Xu et al., 2005; Luo et al., 2007) Prior to visualizing molecules with nanoparticles, fluorescence based labeling was the dominant technique for characterizing immobilized bio-molecules, and is still a useful technique for studies not requiring resolution at the nanoscale (Csaki et al., 2001; Csaki et

al., 2007). Additionally, while optical imaging limitations can be addressed by fluorescing semiconductor nanodots, their potential toxicity to biological molecules requires them to be encapsulated, complicating the bio-conjugation process and lowering the optical fluorescence yield (Wang et al., 2004). In contrast, gold colloids have been highly utilized in biomolecule labeling/visualization assays as they are stable over long periods of time, have readily controlled sizes and are compatible with antibodies, antigen proteins, DNA and RNA (Alivisatos et al., 1996; Mirkin et al., 1996; Hazarika et al., 2007; Yao et al., 2007). Perhaps the most robust form of gold colloid bioconjugation relies on a gold-thiol interaction for the adsorption of thiol-labeled molecules on gold (Csaki et al., 2001; Georgiadis et al., 2000; Petrovykh et al., 2003; Karpovich et al., 1994; Song et al., 2006; Yao et al., 2007). Its well known fast kinetics and single step incubation process reduce contamination risk by avoiding multiple washes (Mo et al., 2005). Chemisorption of gold nanoparticles to biomolecules is typically through free thiol groups available on the analyte which, given the correct stoichiometry, facilitates bio-functionalization of the gold (Taft et al., 2004; Mo et al., 2005; Sato et al., 2003; Csaki et al., 2001). Alternatively, gold nanoparticle modification through terminal amine or carboxyl groups is also common, but additional coupling steps are required (Withey, et al., 2006; Niemeyer et al., 2004).

Using monofunctionalized gold nanoparticles permitted stoichiometric labeling of biomolecules through reaction of an activated succinimide group to amino terminus of proteins and peptides (Ribrioux et al., 1996; Second von Banchet et al., 1995). For the AuNP labeling, the main benefit is that the standard of specificity of a chemical bond (gold-thiolate) is enhanced by the steric requirement of gold having to access a geometrically confined thiol functional group. As the gold nanoparticle size is at 1.4nm, small as a phase contrast reagent in SEM, it would be difficult to image without size enhancement. We used a solution approach to deposit gold ions onto the nanoparticle to reproducibly enlarge the nanogold particles to about 7–8 nm in diameter, resulting in improved backscatter detection of the labeled bioassembly on CNT electrode tips. Gold ions catalytically deposited permits reproducible growth of bioassembly-linked nanoparticle. This linking scenario's specificity is demonstrated by the negligible amount of non-specific CNT sidewall absorption of Npx-AuNP conjugate and the subsequent high affinity of Npx-AuNP present virtually exclusively and stoichiometrically (relative to the bioassembly) on the carboxyl-etched tips of CNTs tips (Figure 2).

This strategy distinguishes itself in that the visualization of successful bioassembly -CNT conjugation is based on imaging chemisorbed gold nanoparticle Npx conjugates linked together via labeled peptide, specifically reacted with the sulfenic acid center of the enzyme, as indicated in Figure 3.

An additional advantage of this approach is that this offers a non-denaturing means of accessing the buried active sites of enzymes. Globular proteins fold such that their reactive centers are protected from nonproductive side-reactions. Consequently, a difficulty in linking modular enzymes to electrodes is penetrating their active centers, where signals are generated, to the electrodes, where these signals are detected. We have circumvented this problem by utilizing peptides that are highly elongated, with a stable and well-defined helical topology that can penetrate into active sites with apparently minimal disruption of conformation, as confirmed by maintenance of enzymatic activity. The activity of Npx was maintained, falling to about 50% of control values when monitored along the Npx-peptide reaction profile, extending the reaction to over three hours (Table 1). It should be noted that this value is skewed negatively due to the nature of the assay and the average functional state of the enzyme is likely higher. The structural integrity of these peptides, confirmed through high resolution structure elucidation recently completed by our group (manuscript in preparation) and by others on leucine zippers (O'Shea et al., 1991) indicates that these can

coordinate metals and maintain distance and geometrical relationships that are conducive to electron transfer. The ease at which peptides can be functionalized highlights the general applicability of this approach to other systems.

Although metallization of peptides is well documented, multiple metallization is less common. This was achieved in the case of the MHP peptide by designing metal-binding sites at outward faces of the strands. These binding sites did not disrupt the critical leucine zipper interaction required for conformational stability (Krantz et al., 2001). Several peptides were synthesized which had variable metal-binding stoichiometries (Yeh et al., 2005). The most efficient transducer of electrons were peptides with multiple binding sites, whereas peptides that bound two metals, one on each end of the peptide, did not transduce any signals, highlighting the importance of maintaining a critical distance between metal centers for electron conduction. Evidence of metal binding to the peptide has been obtained by isothermal titration calorimetry, X-ray absorption fluorescence spectroscopy (EXAF) measurements (Yeh et al., 2005) and most recently by mass spectrometry analysis of the peptide- Co^{2+} complex (Figure 4). For the peptide- Co^{2+} complex formed at pH 9.0, both $[\text{M} + \text{Co}]^+$ and $[\text{M} + 2\text{Co}]^+$ were clearly observed, indicating that at least two cobalt atoms were bound to one peptide strand under basic conditions. It is very likely additional cobalt atoms were bound to the MHP backbone, as indicated by the earlier ITC analysis, but these higher metallization states could not be easily detected by the MALDI-TOF-MS, since the matrix used for MS sample preparation contains trifluoroacetic acid, potentially disruptive to stability of cobalt binding to the histidines of MHP. Nonetheless, these results, in conjunction with the earlier reported solution characterization, strongly indicates metal binding stoichiometries of two to four metals per strand and these are sufficient for electron transduction.

Electrochemical measurements of the Npx-bioassembly linked CNT electrode array showed strong cyclic voltammetry (CV) signals when the metallized peptide nanowire was incorporated (Figure 5, solid line). At a scan rate of 100 mV/s, the anodic peak current is $-22.51 \pm 3.71 \mu\text{A}$ and cathodic peak current is $45.62 \pm 3.76 \mu\text{A}$ for this electrode. A control electrode comprised of CNT electrode linked with Npx-AuNP without peptide as a spacer only gave weak signals (Figure 5, dash line). The effect of scan rate for cyclic voltammetry measurements on the response of the immobilized CNT electrode is shown in Figure 6. The anodic and cathodic peak currents (i_{pa} and i_{pc}) increased in a linearly proportional manner as the scan rate increased from 50 mV to 500 mV (Figure 6). The linearity of response confirms that the signal is generated via immobilized bioassembly on the CNT electrodes and not through solution electroactive species.

4. Conclusion

We have successfully fabricated nanobioassembly arrays comprised of the redox enzyme, Npx, covalently linked to a CNT electrode array through metallized multihistidine peptides based on the sequence of GCN4 leucine zipper. Our results demonstrate that this modularly assembled electrode is a highly sensitive electrochemical biosensor. This biosensor array is capable of detecting changes in redox status *in-situ* (Xia et al, 2006). Evidence from isothermal titration calorimetry, EXAF (Yeh et al., 2005), and MALDI-TOF-MS (this report), shows that the peptide MHP binds to metal ions with micromolar binding affinities. Chemical linkage strategies allow specific covalent linkage of Npx through the disulfide or thioester bond formation between the Cys-thiol or thioester of the peptide and Cys -42 of Npx. Through stepwise formation, the bioassembly of Npx-MHP can also be subsequently covalently linked to CNT electrodes through NHS-assisted amide bond formation. The CNT-bioassembly labeled with gold nanoparticles, whose phase contrast properties were enhanced through a controlled solution deposition approach, can be viewed via SEM. The

SEM results confirmed precise Npx-bioassembly linkage onto the carboxyl-terminated tips of CNT electrode array and not to the sidewalls.

In coordinating the various electron-transducing units of the bioassembly, the metallized peptide served both as a conductor and spacer and was essential in maintaining conformational stability of the enzyme. Our modular assembly method and concept for bioelectrode formation using a peptide as a specific linker and functioning as a nanowire conduit of signals are generalizable and applicable to other bionanoelectrode systems.

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Figure 1.
SEM image taken at a 30 degree tilt angle; scale bar represents 100 nm, of the bare CNT electrode array.

Figure 2.
SEM of CNT Electrode Arrays. A: immobilized bioassembly, localized to CNT tips. B:
Controlled deposition of bioassembly can be achieved to attenuate or amplify output.

Figure 3. Formation of a CNT-Npx electrode through subsequent EDC and NHS conjugation. represents the peptide nanowire.

Figure 4.

MALDI-TOF-MS spectra of peptide MHP binding complex with Co^{2+} in 10 mM Tris/HCl, pH 9.0. $\text{MH}^+ = 4143.11$.

Figure 5. of CNT electrode immobilized with Npx-MHP- Co^{2+} -AuNP (solid line) and with Npx-AuNP (dash line) bioassemblies in 0.1 M potassium acetate buffer, pH 5.4 at a scan rate of 100 mVs^{-1} .

Figure 6.

Plot of peak currents of Cyclic voltammetrys (CVs) of a CNT electrode immobilized with Npx-MHP- Co^{2+} -AuNP vs scan rates. The CVs were measured at scan rates of 50, 100, 200, 300, 400, 500 mV/s respectively in 0.1 M potassium acetate buffer, pH 5.4.

Table 1

Relative Npx activity (normalized against Npx activity in the absence of Guanidine; all measurements have standard deviations of $\pm 18\%$) during Npx and peptide reaction. The reaction buffer contains 50 mM Tris-HCl pH 8.0 and 1.0 M Guanidine.

Time (minutes)	Npx only, in reaction buffer	Npx and H ₂ O ₂ (1:1) in reaction buffer	Peptide MHP added into the mixture of Npx and H ₂ O ₂ at 30 min
2	67%	72%	---
15		70%	---
30	81%	67%	57%, peptide was added at this point.
50	79%	87%	
65			43%
110	70%	72%	32%
210	63%	63%	30%