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13. ABSTRACT ***(Maximum 200 Words)***

The objective of this seed project was the design and integration of bio-molecules with nanoelectronic circuitry, using proteins and novel molecular linkers between the proteins and the nanodevices. Proteins under consideration included redox and photosynthetic proteins, while the potential conductive molecular linkages include conductive DNA with intercalated metallic ions (M-DNA).

The ability to detect sensitively a variety of compounds and integrate the signals into intelligent circuitry is a precursor step to operating such a system in the reverse direction, that is, for the electronically controlled stimulation and release of proteins or other biochemicals to trigger a specific, local and controllable biomolecular reaction. The possibilities of controlling the activation of particular biological events, such as transcription or production of critical mediating proteins in response to detection of mutagens or release of encapsulated antidote antigens upon sensing of toxins, illustrate the additional innovative uses that can result from this project.

This seedling project formed the foundation for our team to launch parallel and collaborative research threads aimed at addressing the key challenges and demonstrating several linker technologies for a range of target biomolecules.

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Engineering DNA for interfacing redox protein with read-out

Seedling project report to DARPA DSO (Dr. A. Krishnan) (Agent: AFOSR, Hugh De Long)

Grant #: F49620-02-1-0341

Summary

The objective of this seed project was the design and integration of bio-molecules with nanoelectronic circuitry, using proteins and novel molecular linkers between the proteins and the nanodevices. Proteins under consideration included redox and photosynthetic proteins, while the potential conductive molecular linkages include conductive DNA with intercalated metallic ions (M-DNA).

The ability to detect sensitively a variety of compounds and integrate the signals into intelligent circuitry is a precursor step to operating such a system in the reverse direction, that is, for the electronically controlled stimulation and release of proteins or other biochemicals to trigger a specific, local and controllable biomolecular reaction. The possibilities of controlling the activation of particular biological events, such as transcription or production of critical mediating proteins in response to detection of mutagens or release of encapsulated antidote antigens upon sensing of toxins, illustrate the additional innovative uses that can result from this project.

This seedling project formed the foundation for our team to launch parallel and collaborative research threads aimed at addressing the key challenges and demonstrating several linker technologies for a range of target biomolecules.

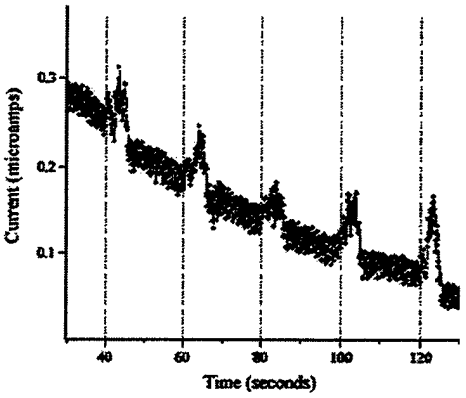
The successes arising from this seed project include the following:

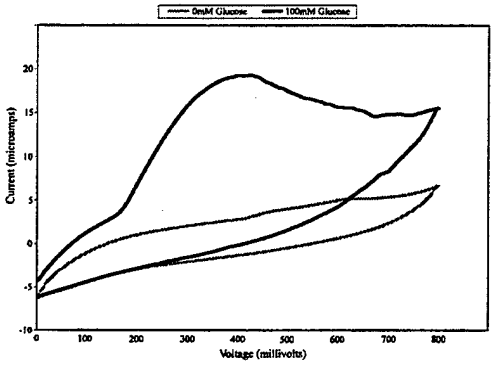
1. linking glucose oxidase (GOx) to carbon nanotube array electrodes and recording electron transfer in real-time.
2. Development of a novel platform and approach for directing GOx to and covalently binding it only to the tip of the carbon nanotube, thereby obtaining an electron transfer rate of several thousand per second, greatly exceeding previously recorded levels.
3. Initial evidence of success in linking DNA to a designed peptide and then converting the DNA to metallic DNA.
4. Designed peptides as linking agents between target enzymes and conductive electrodes.

Accomplishments and New Findings from this Seedling project

Glucose oxidase–carboxyl link–carbon nanotube system

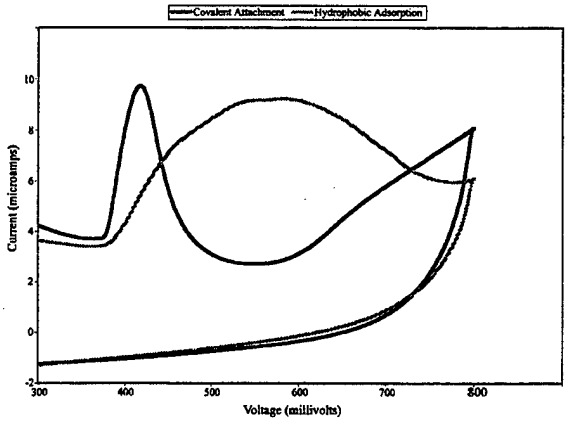
As a proof-of-concept study, during this project, the group at Brown covalently modified a highly-ordered carbon nanotube (CNT) array electrode with glucose oxidase and placed it in a flow cell under a near-constant flow of buffer solution. After determining the baseline current at a constant potential of 500 mV, a glucose solution was injected at timed intervals, and the resulting rise in current was measured[1]. The current arose from the two-electron oxidation of glucose by the immobilized glucose oxidase. The system was also stable over a wide range of pH (4.0–9.0).



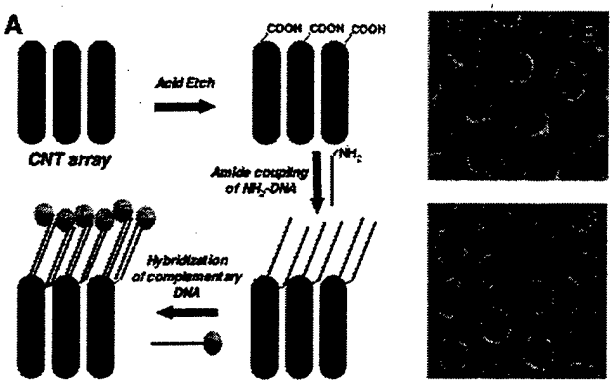


On a similarly prepared CNT electrode, cyclic voltammetry (CV) was performed to further characterize the GOx-CNT conjugated system. A very distinct peak occurred around 400 mV, which is the expected redox potential of GOx. Various glucose concentrations were used in the study, and a direct relationship was found between concentration and CV response. This result shows the promise of this system for protein biosensor applications.

The group then examined the properties of the protein-CNT conjugate with two different methods of protein immobilization. On one CNT electrode, glucose oxidase was covalently bound to the tips, and the hydrophobic adsorption to the side-wall was prevented by the use of a surfactant. On another electrode, the protein was allowed to adsorb to the walls of the tubes, but the tips were capped so that no covalent bond could be made. CV was then performed on the two electrodes. It was found that the protein linked to the tips gave a very sharp peak at 400 mV, while the protein adsorbed to the side-walls gave a much broader peak extending from 400–700 mV. This, we expect, is due to the fact that the adsorbed protein exists over a range of somewhat denatured states, and is therefore less active than the protein linked to the tips, which is still in its active conformation. Furthermore, in terms of the amount of protein bound (based on available surface area for attachment) the adsorbed protein outnumbers the covalently-linked protein by more than an order of magnitude, but the current response of each



species is almost identical. This shows not only the importance of preserving the natural active conformation of the protein, but also the advantage of linking to the tips, where electron transfer is intrinsically more efficient than the side-wall.



(A) Procedure used to functionalize CNTs. (B) SEM image of CNT array derivatized with amino-DNA and then subjected to hybridization with a complementary strand modified with a 10 nm gold nanoparticle. (C)

Functionalization of CNT arrays for pilot electrochemical studies

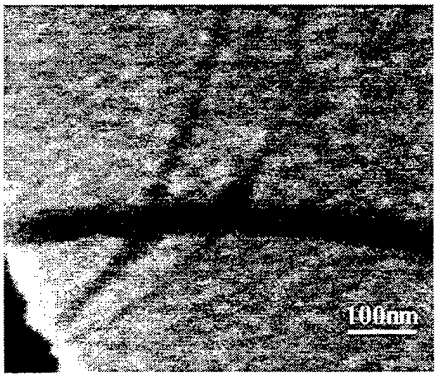
In a joint effort between the **Xu** group and the group of Professor S. O. Kelley at Boston College, vigorous efforts building on the Seedling project, are underway in the greatly expanded scope of a MURI program to functionalize CNT arrays with DNA oligonucleotides. The covalent attachment of synthetic DNA will permit the targeted delivery of biomolecular receptors that will serve as sensing elements.

We have adopted techniques from Alivisatos[2] to form conjugates of single stranded (SS) DNA and gold nanoparticles. Conjugates are sorted by gel electrophoresis with incrementally varying mole ratios, and the gel transit lengths are compared to those of control lanes to verify that linking has indeed taken place.

To monitor the conjugation of amino-terminated DNA molecules, we have developed an assay that permits direct visualization of the hybridization of a complementary sequence. The complement is labeled with a 10 nm Au nanoparticle which is visible when the CNT array is visualized by SEM. As shown in the schematic diagram, the CNTs are first acid treated to produce free carboxylic acids at the tips, and amino-functionalized DNA is then introduced in the presence of reagents that facilitate the formation of a peptide bond. Non-covalently bound DNA and coupling agents are washed away from the array, and Au-labeled DNA is then introduced.

Linking the DNA molecules to the Au nanoparticles represents the first part of this final step. The protocol involves mixing micro-molar quantities of stabilized gold nanoparticles into solutions of micro-molar thiolated SS-DNA and milli-molar divalent cations as described by Mirkin[3].

The final step in this process is linking the DNA molecules to nanotubes. We have linked B-DNA (>10um) non-specifically to CNTs as described in the



SEM image of carboxylated CNT linked to B-DNA

work of Dekker[4]. However, this technique does not rely on surfactants (SDS/GA) due its potentially deleterious effect on electrical transport through the DNA-CNT complex. By first aligning the CNTs across planar electrodes with an radio-frequency electric field, the CNTs are annealed to hold them in position and then oxidized via procedures reported by Smalley[5]. Subsequently, the nanotubes are linked to DNA through amine modified 'sticky ends' of lambda phage DNA.

By hybridizing complementary thiol and amine modified SS-DNA we use self-assembly to create Au nanoparticle-DNA-Nanotube complexes. Amine modified SS-DNA covalently bonds to carboxyl groups on the CNT surface while thiolated SS-DNA covalently bonds to gold. Further investigations are underway to mitigate non-specific absorption of SS-DNA hydrogen bonding to the CNT electronic platform.

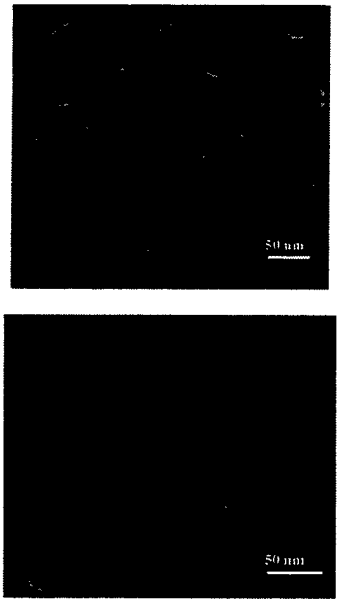
The electron micrography below shows the results of an experiment conducted using this sequence of steps. On an array that was treated with amino-DNA, Au nanoparticles are observed around the periphery of the CNTs. On the array that was treated identically except for the omission of DNA from the coupling mixture, Au nanoparticles do not accumulated in significant amounts around the CNTs.

We are now investigating the sensitivity of this assay to mismatches within the immobilized duplex to evaluate how to use encoded sequence information to self-assemble sensor modules on the arrays. We are also exploring the use of CNTs as nanoelectrodes in solution electrochemistry experiments.

M-DNA—a molecular conductor

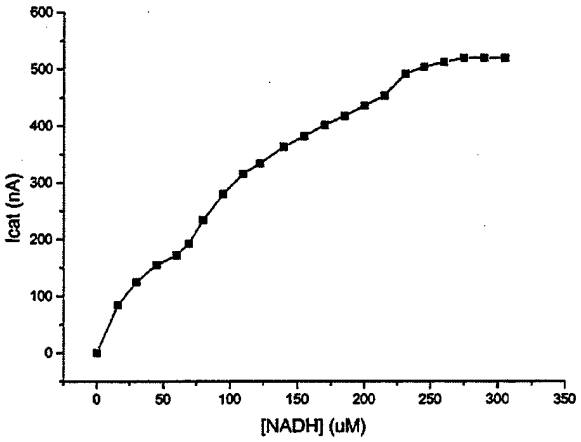
We have been able to convert B-DNA to metallic DNA (M-DNA)[6] using zinc, cobalt or nickel ions to replace imino protons of the guanine and thymine base pairs in a tris-buffer solution of pH 8. Nickel cations give the most stable form of M-DNA, followed by cobalt and then zinc. The base sequence also has an affect on stability – the most stable being poly-GC.

The conversion then takes place by titrating B-DNA into a 20mM tris-buffer solution (pH 8.5) to a concentration of 50μg/ml. The lowest acceptable pH for M-DNA buffers is pH 8.0. The metal cation is then added ($NiCl_2$, $Co\ Cl_2$, $Zn\ Cl_2$) to a final concentration of 100mM. The solution is then left at room temperature for 20 minutes.



(A) SEM image of gold nanoparticle labeled complementary DNA on a highly ordered nanotube electrode (B) SEM image of control sample with gold nanoparticle labeled non-complementary DNA

After forming M-DNA, it is necessary to verify that the new form has been created. One way to test for successful M-DNA conversion is an ethidium assay. Adding ethidium to B-DNA gives rise to a clear fluorescence signal around 600nm. However, adding ethidium has no effect on M-DNA, since M-DNA does not permit the ethidium to intercalate, and therefore yields a very weak fluorescence. For our experiments, an ethidium bromide concentration of 0.5 μg/ml is ideal.



Maximum peak current plot derived from the stepwise addition of NADH until saturation. The value of the peak current along with the number of assembly molecules on the electrode surface was used to obtain the first order electron transfer rate constant, ~3000 s^{-1}.

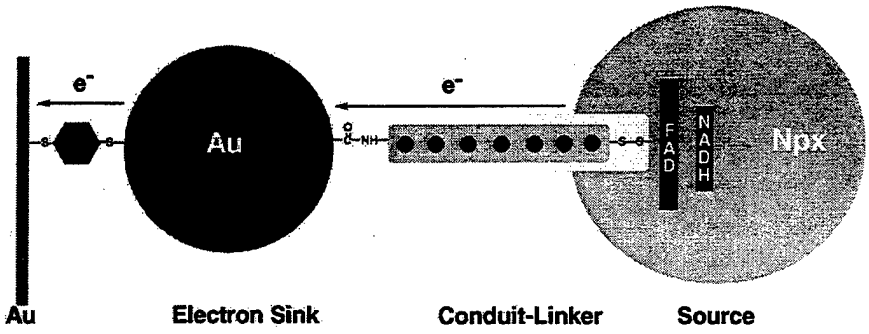
Another way to test for the successful conversion to M-DNA is by using DNase. DNase is an enzyme that cleaves B-DNA, but not M-DNA. By measuring the fluorescence of the DNA before and after the addition of DNase, one can verify the conversion to M-DNA. The conversion has been successful if the fluorescence does not change with the addition of the enzyme.

This novel molecular conductor could provide a basis for high-conductivity molecular linkages in biochemical electronic systems like our proof of concept experiment on glucose oxidase. Through hybridization of DNA and subsequent conversion to M-DNA, precise placement of molecular-scale conducting wires may be achieved.

NADH Specific Signal Amplification through an Electrochemically Active Biocatalytic Assembly

The **Co-PI Joanne Yeh** and her group achieved valuable results during this Seedling project. In particular, a molecular assembly consisting of a redox enzyme, a metallized double-helical peptide, and a gold nanoparticle immobilized onto a gold wire, derivatized with a phenyldithiol compound initiated and conducted redox signals in the presence of H_2O_2 and NADH. The current generated by the binding of NADH, the electron donor, was transduced through the molecular assembly with little to no loss of signal to the solution. The currents measured correlate to an electron transfer rate constant on the order of 3000 s^{-1}. This electron transfer rate is two orders of magnitude faster than the endogenous electron transfer rate from NADH to the native enzyme, 27 s^{-1}. This rate indicates that the metallized peptide is in an optimal conformation for electron transfer, and, in conjunction with the nanoparticle, is an effective

conduit of electrical signal. The Au nanoparticle, acting as a relay between the metallized peptide and the bulk electrode effectively directs the current from the redox enzyme to the electrode.

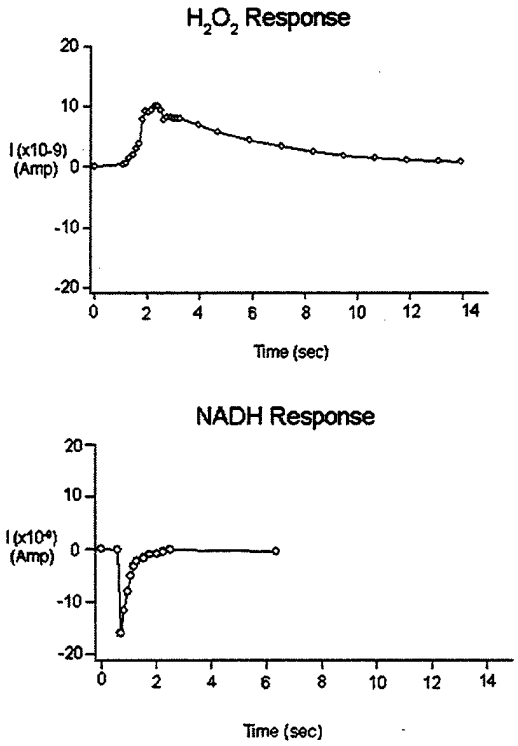


Assembly of AuNP-Co-MHP-Npx electrode through stepwise linkage of each component with adsorption of the AuNP linked complex reconstituted onto a dithiol monolayer associated with a Au electrode. AuNP linked complex reconstituted onto a dithiol monolayer associated with a Au electrode.

Formation of semiconductor nanoparticles based on native biological macromolecules sensitive to external stimuli is an attractive system for a variety of applications[7,8]. The use of redox enzymes as the source of electronic signals that can be propagated efficiently in response to a specific ligand or other physiological signal has many potential applications, such as amperometric biosensors[9,10]. A major limitation in using biomolecules in electrodes is that the incorporation of these molecules is complicated by a lack of pathways that can transport electrons from their embedded redox sites to an electrode. Although electrical communication between redox proteins and electrode surfaces has been improved through various approaches[11,12,13], these methods contact the enzyme at somewhat random conformations with respect to the electron relaying units, ultimately resulting in decreased rates of electron transfer. To produce electroactive biocatalysts in amperometric biosensors with sufficient kinetics of electron transfer, the relaying units must be linked in a controlled and conformationally defined manner[14,15,16,17].

The harnessing of electrons is through a modularly formed, redox active assembly that is immobilized with gold nanoparticles onto a bulk 1,4-benzenedithiol derivatized -Au electrode.

Molecular Assembly: MPeptide-Enzyme-AuNP



Electrode surface : 1.1x10^{-15} mol/cm^2 ~ nA signals

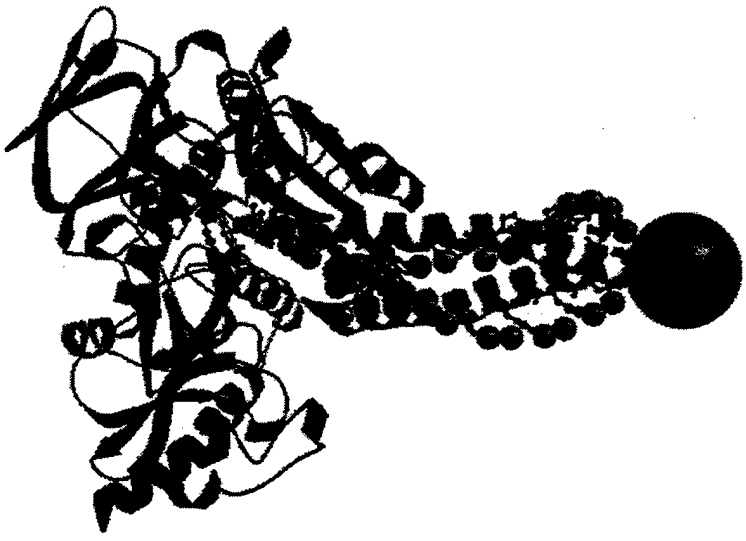
Current design: 10^{-12} mol/cm^2 ~ µA signals

Plots of the currents generated from addition of H_2O_2 and NADH versus time. H_2O_2 results in surface and cobalt oxidation, nonspecific to the enzyme while NADH is dependent on enzyme binding, exhibiting fast time constants with opposite current flows, as expected.

The system is comprised of a redox-active enzyme, a bacterial NADH peroxidase (Npx), a 49 kD bacterial flavoenzyme that catalyzes the two electron reduction of $H_2O_2 \rightarrow H_2O$. This reduction is initiated upon binding of the cofactor NADH, which donates 2-electrons through the primary redox center, FAD. Under oxidizing conditions, the electrons are passed onto a molecule of peroxide, bound to the secondary redox center, a thiolate residue. This residue progresses from the reduced thiolate ($-S^-$) to the oxidized sulfenate ($-SO^-$) states during the catalytic cycle[18]. The three-dimensional crystal structures of WT, oxidized and reduced forms of Npx[19] have been solved to atomic resolution so that the precise conformation of the active site residues and steric limitations within the active site are well-defined.

Based on the unique cysteine-sulfenic acid chemistry utilized by Npx at the redox center, we designed a metallizable peptide (MHP), bifunctionalized so that each terminus allowed linkage to a specific components of the signaling assembly and of sufficient length to penetrate the active site. A sulfo-N-hydroxysuccinimido group on the Au nanoparticle forms a link to the MHP by nucelophilic attack on the NT primary amine group of the peptide. Prior to formation of the peptide-Au nanoparticle complex, cobalt-metallization of the peptide was achieved by incubation of the MHP with a $CoCl_2$, forming a fully substituted metallized peptide (Co-MHP). Once the Au nanoparticle-Co-MHP is reacted with the Npx enzyme, a disulfide bond forms between the CT cysteine of the peptide to the enzymatically active cysteine of Npx.

For the final reconstitution of Npx-CoMHP-Au nanoparticle assembly onto the Au electrode, a self-assembled monolayer was formed by adsorption of 1,4-benzenedithiol (BDT) on Au wires. This provided the reaction surface for final immobilization by reaction of the Au nanoparticle with thiols of BDT monolayer (see schematic).



Model of the assembly based on the atomic coordinates of the Npx and MHP. The peptide coordinates cobalts (small grey sphere) through histidine residues at every i, i+4 positions. AuNP was modeled in as a sphere, to scale with the biological molecules. with a diameter of 14 A.

An advantage to this system is that in addition to the atomic resolution structures of Npx, the kinetics of this enzyme have been well-characterized so that the rate constants of several steps in the reaction cycle are known. This information helps to correlate the electron transfer rate constant derived from the currents measured in this system to the native reaction rates and allows an assessment of the efficiency of the designed assembly in electronic signal transduction.

Based on reaction rate enhancements reported due to proximity and orientation effects in native enzyme and receptor systems, a coordinated assembly whose components are optimally tethered on an electrode would be expected to enhance rates of reactions by up to 100–fold. Furthermore, the tethering of a signaling assembly containing the redox enzyme onto an electrode could provide enhancement of electronic signal transduction rates by minimizing loss of signal to the solution. Particularly relevant to the formation of a bioelectrocatalytic system is the spatial orientation of the signaling unit and all its components, which must to be reconstituted in a conformation that is conducive for charge transfer. Here we report the formation of a redox-active assembly whose electron transfer rates are increased by 100-fold compared to the rate of unimolecular enzyme reduction by NADH, the electron donor (Figure above and below). Furthermore, these results suggest that the cobalt-metallized peptide bridge between the redox enzyme and the AuNP bulk electrode is an efficient conduit of electrical signals that provides a stable, conformationally rigid link which can be adapted for use in other systems.

The efficiency, specificity, and modularity of biomolecules such as proteins and nucleic acids make them attractive material for designing highly sensitive and intelligent circuitry. Utilization of natural macromolecules as sensors exploit the inherent recognition and diversity of these molecules. Effective integration of biomolecules with nanoelectronic circuitry holds great promise for the design of compact and highly sensitive systems.

Our metallized peptide appears to be an efficient mediator of electrons. The precise mechanism by which electrons are propagated through the peptide remains to be studied although based on the 5.2 Å between Co modeled into the peptide, a hopping mechanism seems more likely than through-bond transduction. The use of designed peptides as conduits of electronic signals through metallization can be used in a variety of systems, allowing convenient use of these as a modular conduit of electronic signal, especially since functionalities are readily incorporated for specific linking reactions.

Linking of biomolecules to electronic circuitry can be essential to the design of efficient signal transduction assemblies in bioresponsive systems. The utilization of metal nanoparticles that act as an electrical nanoplug helps to align the enzyme on the conductive support. In our work, the AuNP appears to channel the current to the electrode to help maximize the efficiency of electron transfer. Direct electronic coupling between electrodes and enzymes has been achieved in our assembly and current measurements suggest that the assembly mediates electron transfer with very high efficiencies.

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[1] Withey, G. D., Lazareck, A. D., Tzolov, M. B., Yin, A., Yeh, J. I., Aich, P., and Xu, J. M., submitted to *Nature Biotechnology, in review*

[2] Alivisatos, A. P. et. al., *Nature.* 382, 609 (1996)

[3] Mirkin C. A. et. al., *Nature.* 382, 607 (1996)

[4] Dekker C., et. al., *Nature.* 420, 761 (2002)

[5] Smalley, R. E. et. al., *Journal of Physical Chemistry B.*105, 2525 (2001)

[6] Rakitin, A., Aich,P., Papadopoulos,C., Kobzar, Yu., Vedeneev,A.S., Lee,J.S. and Xu, J. M., "Metallic conduction through engineered DNA: DNA nanoelectronic building block", *Phys. Rev. Lett.*, **86**, 3670 (2001).

[7] Anderson, J.L., Bowden, E.F., & Pickup, P.G. *Anal. Chem.* **68**, 379R-444R (1996).
[8] Hamad-Schifferli, K., Schwartz, J.L., Santos, A.T., Zhang, S., and Jacobson, J.M. (2002). Remote electronic control of hybridization through inductive coupling to an attached metal nanocrystal antenna. *Nature* **415**, 152-155.
[9] Taton, A.T., Mirkin, C.A., & Letsinger, R.L. Scanometric DNA array detection with nanoparticle probes. *Science* **289**, 1757-1760 (2000).
[10] Wang, J. Amperometric biosensors for clinical and therapeutic drug monitoring: a review. *J. Pharm. Biomed. Anal.* **19** (1-2), 47-53 (1999).
[11] Schuhmann, W., Ohara, T.J., Schmidt, H.-L., & Heller, A. *J. Am. Chem. Soc.* **113**, 1394 (1991).
[12] I. Willner et al. *J. Am. Chem. Soc.* 118, 10321 (1996).
[13] Rajagopalan, R., Aoki, A., & Heller, A. *J. Phys. Chem.* 100, 3719 (1996).
[14] Xiao, Y., Patolsky, F., Katz, E., Hainfeld, J.F., & Willner, I. "Plugging into Enzymes": nanowiring of redox enzymes by a gold nanoparticle. *Science* **299**, 1877-1881 (2003).
[15] Medvedev, D.M., Daizadeh, I., & Stuchebrukhov, A.A. Electron transfer tunneling pathways in Bovine Heart Cytochrome C oxidase. *J. Am. Chem. Soc.* **122**, 6571-6582 (2000).
[16] Scott, D.L. & Bowden, E.F. Enzyme-Substrate Kinetics of Adsorbed Cytochrome C Peroxidase on Pyrolytic Graphite Electrodes. *Anal. Chem.* **66**, 1217-1223 (1994).
[17] Nahir, T.M., Clark, R. A., & Bowden, E.F.. Linear-sweep voltammetry of irreversible electron transfer in surface-confined species using the Marcus theory. *Anal. Chem.* **66**, 2595-2598 (1994).
[18] Poole, L.B. & Claiborne, A. Interactions of pyridine nucleotides with redox forms of the flavin-containing NADH peroxidase from Streptococcus faecalis. *J. Biol. Chem.* **261**, 14525-145233 (1986).
[19] Yeh, J.I., Claiborne, A., & Hol, W.G.J. Structure of the Native Cysteine-sulfenic acid redox center of enterococcal NADH peroxidase refined at 2.8 Å resolution. *Biochemistry* **31**, 9951-9957 (1996).