

**JES FOCUS ISSUE IN RECOGNITION OF ADAM HELLER AND HIS ENDURING CONTRIBUTIONS TO ELECTROCHEMISTRY****Controlled Placement of Enzymes on Carbon Nanotubes Using Comb-Branched DNA**Nina Z. Hausmann,^a Shelley D. Minteer,^{b,*} and Dana A. Baum^{a,**,z}^aDepartment of Chemistry, Saint Louis University, St. Louis, Missouri 63103, USA^bDepartment of Chemistry, University of Utah, Salt Lake City, Utah 84112, USA

Immobilization is not only useful for preserving enzyme activity, but also to adhere an enzyme to a surface, such as an electrode, so that the enzyme does not leach into solution during testing. Current immobilization approaches do not readily allow for adjustments to the distance between the enzyme and the electrode or other enzymes. The ability to control the distance of enzymes relative to each other on an electrode can allow for optimal placement and improved current responses. In this report, we investigate the use of comb-branched DNA for enzyme immobilization. A DNA foundation strand was covalently attached to multiwalled carbon nanotubes on a glassy carbon electrode. Comb-branched DNA was then successfully formed using a previously-identified deoxyribozyme to attach DNA strands at specific locations on this foundation strand. By changing the foundation strands, the placement of the DNA strands can be adjusted, allowing for distance changes between the enzyme and the electrode surface. Using standard bioconjugation methods, alcohol dehydrogenase and glucose dehydrogenase were attached to these comb-branched DNA structures, resulting in enzyme immobilization on electrode surfaces. Amperometric analysis revealed both distance and DNA foundation strand length dependence for current response of these enzymes in the presence of their appropriate substrates.

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In biosensor and biofuel cell applications, immobilization of the biocatalysts onto a surface, such as an electrode, is a crucial step in developing functional and effective devices. Enzymes in particular have been widely used in these applications due to their high specificities and for their ability to provide desired high current and power densities.¹ Enzyme immobilization is not only useful for preserving the activity of the enzyme, but also for the physical aspect of adhering an enzyme onto an electrode so that the enzyme does not leach into solution during operation. Assuming other components of the electrode are non-reactive with the substrate of interest, loss of the enzyme from the electrode results in loss of current response or power output.

There are a variety of reported immobilization methods, including adsorption, entrapment in sol-gels, encapsulation in polymers, and chemical cross-linking.^{2,3} However, many of these techniques do not provide options for controlling the distance between the enzyme and a surface or between the enzyme and other enzymes. The ability to adjust these distances in a controlled manner can improve current devices by allowing for optimization of enzyme placement.

In addition to the methods above, enzymes have also been immobilized using DNA. For example, thiolated strands of DNA can be directly attached to the enzyme or can be covalently attached to a cofactor to capture an enzyme. These thiolated complexes can then be immobilized on gold electrodes for amperometric studies.¹ While changing the length of the DNA strand provides some control over the distance between the enzyme and the surface, the enzyme loading on the surface is limited by monolayer formation, which restricts maximum current density of electrodes. DNA structures, rather than just simple single strands, could provide additional options for placement control.

A type of DNA structure that could be used for controlled enzyme immobilization is comb-branched DNA. Comb-branched DNA consists of one or more nucleotides that have 3 oligonucleotide strands emanating from the 5', 3', and 2' position of the sugar.⁴⁻⁷ While comb-branched DNA can be formed via chemical synthesis^{5,8,9} or photoligation,¹⁰ both of these approaches require specialized nucleotide building blocks. A simpler approach uses catalytic DNA, or deoxyribozymes, to attach adenylated DNA strands to ribonucleotides in a DNA foundation strand using components that are commercially

available or readily prepared in the lab.^{6,7} In addition, the deoxyribozyme approach allows for several branches to be formed on a single foundation strand, with each branch strand being a different sequence if desired.⁶

There are several advantages to using comb-branched DNA for enzyme immobilization. The distance between the attachment points on the foundation strand can be adjusted so the separation between the DNA branch strands changes in a defined way. This provides the ability to control the distance from the surface and for attachment at several different sites along the DNA foundation strand. Each DNA branch strand on the foundation strand can serve as an enzyme attachment point, allowing for multiple enzymes to be immobilized on a single piece of DNA. If working with a single enzyme, the increased enzyme immobilization can improve the sensitivity and current density of a biosensor or a biofuel cell by increasing catalyst loading. Different enzymes could also be attached to a single foundation strand. Attaching two or more enzymes involved in an electron transfer cascade will also increase current and power outputs for biofuel cells or increase sensitivity for biosensors.¹¹ Finally, the length of the DNA branch strands can be changed to offer another possible point of distance adjustment.

A strategic method was developed to attach these comb-branched DNA-enzyme structures on multi-walled carbon nanotube (MWCNT)-functionalized electrodes. Figure 1 depicts the final components of the electrodes. Amperometry was used to evaluate the current response of the enzymes immobilized on the electrode using comb-branched DNA. The two enzymes that were immobilized independently on electrodes are NAD⁺-dependent alcohol dehydrogenase (ADH) and glucose dehydrogenase (GDH). ADH^{12,13} and GDH¹⁴ are widely used enzymes in the biosensor and biofuel cell area and both have relatively high specific activities (>200 U/mg), are relatively stable at room temperature and neutral pH (or higher), and have well-known bioelectrochemistry. Furthermore, these two completely different enzymes were used to investigate if this immobilization approach can be a general approach for more than one specific enzyme.

Materials and Methods

Materials and reagents.— Potassium phosphate, sodium phosphate, sodium bicarbonate, sodium nitrate, Tris, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), nicotinamide adenine dinucleotide (NAD⁺), NAD⁺-dependent alcohol dehydrogenase, NAD⁺-dependent glucose

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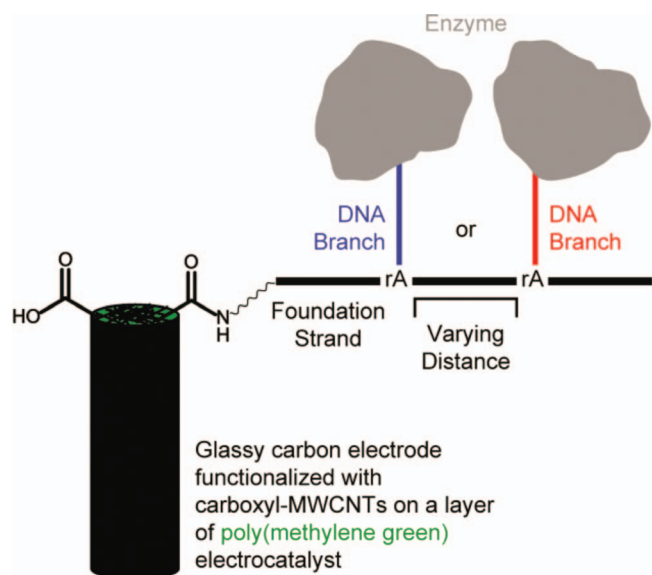


Figure 1. Electrode functionalization strategy using comb-branched DNA. A layer of poly(methylene green) was electropolymerized on a polished glassy carbon electrode. Next a layer of COOH-MWCNTs was pipetted on the poly(methylene green) layer to form a film. The amino-modified DNA foundation strand containing two riboadenosines (rA) for branch attachment was coupled to the COOH-MWCNTs using 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium (DMT-MM). The distance between the branch points was adjusted in each tested foundation strand by inserting nucleotides between the two rAs, resulting in different foundation strand lengths. The DNA branches were attached to the rA using a deoxyribozyme, 8LV13.⁷ Each branch strand had a different sequence and was directed to a specific branch point. For these studies, only single branches were tested. The final step involved coupling the enzyme, either ADH or GDH, to an amine on the DNA branch strand using DMT-MM.

dehydrogenase, glucose, ethanol, and manganese chloride were purchased from Sigma Aldrich. Carboxylic acid-modified multi-walled carbon nanotubes (MWCNTs) were purchased from CheapTubes, Inc. 4-(4, 6-Dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium (DMT-MM) was purchased from Fluoro Chem. T4 DNA ligase and adenosine-5'-triphosphate (ATP) were purchased from Fermentas and Research Products International, Corp., respectively. Phenol/chloroform/isoamyl alcohol mixture was purchased from Fisher Scientific. All reagents were used as received.

DNA and enzyme quantification.— All DNA oligonucleotides were ordered from Integrated DNA Technologies (IDT, Coralville, IA, USA). Oligonucleotides were then purified using either denaturing PAGE or phenol:chloroform:isoamyl alcohol (PCI) extraction. Following ethanol precipitation, DNA recovery was quantified spectroscopically using a NanoDrop 2000c spectrophotometer. Enzyme quantification was performed using the standard Coomassie (Bradford) protein assay.¹⁵ Bovine serum albumin (BSA) concentrations ranging from 62.5–2000 $\mu\text{g/ml}$ were used to generate the standard curve.

Adenylation reactions.— To perform the branching reactions needed for the formation of comb-branched DNA, 5' adenylation DNA substrates are necessary. The DNA strands for adenylation were 5'-pGGAAGAGATGGCGACGG-NH₂-3' (D135) and 5'-pGGAAGATCGTTATCATT-NH₂-3' (D136). Each strand contained a 5' phosphate group and a 3' tethered amine. The adenylation was performed using a published procedure¹⁶ as discussed in Supporting Information.

Electrode preparation.— Experimental details concerning electrode preparation and functionalization are provided in Supporting In-

formation. Briefly, methylene green was electropolymerized onto polished 3 mm glassy carbon electrodes (GCE). Next, COOH-MWCNTs were cast onto the poly(methylene green) layer to form a MWCNT film for DNA attachment. The foundation strands were coupled to the MWCNT-coated electrodes using DMT-MM and branched DNA synthesis was achieved using 8LV13 deoxyribozymes.⁷ The final step of electrode preparation involved coupling either alcohol dehydrogenase or glucose dehydrogenase to the comb-branched DNA using DMT-MM.

Three different foundation strands were investigated and had the following sequences:

D134: 5'-NH₂-GGATAATACG(rA)CTCACTGCGGGAGCCGC-AG(rA)CTCACTGCG-3'; D151: 5'-NH₂-GGATAATACG(rA)CTCACTGCG(CAA)₆GGAGCCGCAG(rA)CTCACTGCG-3'; and D152: 5'-NH₂-GGATAATACG(rA)CTCACTGCG(CAA)₁₂GGAGCCGC-AG(rA)CTCACTGCG-3'. Each sequence contained a 5' tethered amine and two adenosine ribonucleotides (rA) embedded in the sequence. The location of the rA closest to the 5' end of the foundation strand was fixed in all foundation strands. The distance of the rA furthest from the 5' end was increased in each foundation strand by introducing CAA repeats between the rAs. Substrate D135 was ligated to the rA located closest to the 5' end of the foundation strand while substrate D136 was ligated to the rA located furthest from the 5' end of the foundation strand.

Amperometric analysis of enzyme kinetics.— Prior to studies with the enzyme-modified electrodes, amperometric NADH concentration studies (Figure S1) and spectroscopic activity assays of ADH and GDH were performed to determine optimal buffer conditions for amperometric enzyme substrate concentration studies (Figure S2). For ADH studies, the optimal buffer was 100 mM sodium phosphate buffer pH 8.5, containing 100 mM sodium nitrate and 1.5 mM NAD⁺. Injections of 100% ethanol were used to obtain amperometric responses for varying ethanol concentrations. For GDH studies, the optimal working buffer was 100 mM potassium phosphate buffer pH 8.5, containing 100 mM sodium nitrate and 1.5 mM NAD⁺. A 1 M glucose solution was used for injections to obtain amperometric responses for varying glucose concentrations. Amperometric measurements were obtained by applying a constant potential of 0.3 V versus SCE with a platinum gauze counter electrode. The same studies were performed with all appropriate control electrodes.

Results and Discussion

Amperometric concentration studies were performed with modified electrodes and the corresponding controls. Three different foundation strands with varying lengths were tested: D134 (40-mer), D151 (58-mer), and D152 (76-mer). The distance for the D135 branch, which is roughly 3.5 nm from the nanotube did not vary among the three different foundation strands; however, the D136 branch site location along the foundation strand was varied. The D136 branch distances from the nanotube along the foundation strand were approximately 10 nm, 16 nm, and 22 nm in D134, D151, and D152, respectively (Figure S3). The amount of enzyme attached to each electrode was approximately the same for all electrodes (Supporting Information).

The results obtained indicate that the location of the branch relative to the nanotube and the length of the foundation strand play important roles in enzyme current response (Figure 2). For ADH, the observed current and V_{max} decreased as the distance between the enzyme and the nanotube was increased. When ADH was attached to the closest branch (D135) on the shortest foundation strand (D134), we observed a V_{max} of $2.23 \pm 0.08 \mu\text{A}$ (Table I). Moving the enzyme approximately 3.4 nm further from the nanotube to the D136 branch leads to a drop in V_{max} to $1.38 \pm 0.03 \mu\text{A}$. An increase in foundation strand length is required to move the D136 branch further from the nanotube. This increased length also has an effect on V_{max} . When we kept ADH at a fixed location (D135 branch), we saw a clear decrease in V_{max} with increasing foundation strand length (Table I). A similar result was

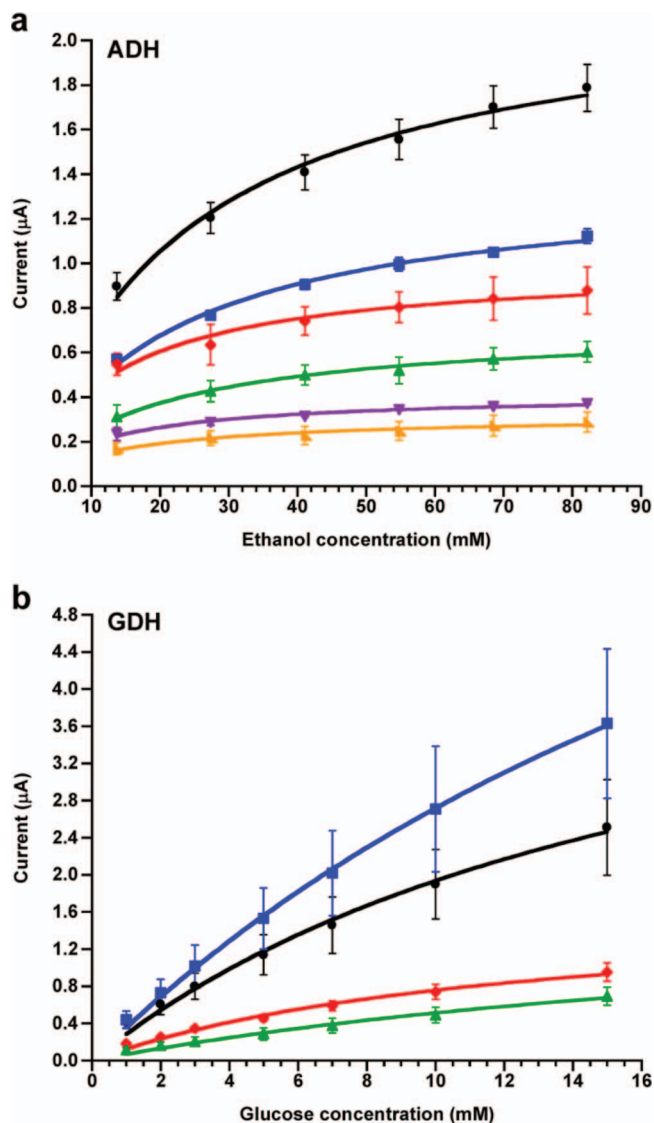


Figure 2. Amperometric concentration studies at 0.3 V versus SCE using modified electrodes containing (a) ADH or (b) GDH coupled to comb-branched DNA. Electrodes were modified with polymethylene green and carboxylated MWCNTs, followed by attachment of one of three foundation strands, D134, D151 or D152. Branch strand D135 was attached close to the nanotube (~3.4 nm), while D136 was attached further away at ~10 nm (D134), ~16 nm (D151), and ~22 nm (D152). ADH was tested in 100 mM sodium phosphate buffer pH 8.1, 100 mM sodium nitrate, and 1.5 mM NAD^+ with increasing concentrations of ethanol. GDH was tested in 100 mM potassium phosphate buffer pH 8.5, 100 mM sodium nitrate, and 1.5 mM NAD^+ with increasing concentrations of glucose. Tested comb-branched structures were D134 + D135 (black), D134 + D136 (blue), D151 + D135 (red), D151 + D136 (green), D152 + D135 (purple), and D152 + D136 (gold).

obtained for control electrodes that were functionalized with each foundation strand without the branches and with either ADH or GDH (Figure S4). Because the enzyme yield was similar for all ADH and GDH electrodes, this drop in current is not due to the amount of enzyme attached. The most likely cause of this current decrease is an interaction between the foundation strand DNA and the MWCNT surface. Specifically, the DNA could be coiling or wrapping around the nanotubes. The longer the DNA, the more it can coil and act as a resistive layer on the electrode surface. Consequently, a decrease in current response is observed. V_{max} continues to drop as the D136 branch is moved further away from the electrode, most likely due to a combination of these factors. (Table I).

Table I. V_{max} and K_m values for immobilized enzymes on different foundation strands at different branch points.

Enzyme	Foundation strand	Branch strand	V_{max} (μA)	K_m (mM)
ADH	D134	D135	2.23 ± 0.08	22.4 ± 2.4
		D136	1.38 ± 0.03	20.9 ± 1.7
	D151	D135	0.99 ± 0.04	12.9 ± 2.2
		D136	0.73 ± 0.02	19.0 ± 1.9
	D152	D135	0.42 ± 0.01	11.5 ± 1.3
		D136	0.32 ± 0.02	13.5 ± 2.8
GDH	D134	D135	5.41 ± 0.70	17.9 ± 3.6
		D136	10.5 ± 0.93	28.6 ± 3.5
	D151	D135	1.70 ± 0.19	12.5 ± 2.4
		D136	1.85 ± 0.44	26.0 ± 8.6

GDH did not show the same trend for different foundation strands (Figure 2B). When attached to the shortest foundation strand, the current was higher for GDH attached to D136, about 10 nm from the nanotube, compared to the closer attachment of D135 (Table I). However, when the foundation strand was lengthened to move D136 further away, almost no difference in V_{max} was observed between the two branch locations.

While V_{max} values varied with branch location and foundation strand length, the K_m values were all within the same order of magnitude for each enzyme (Table I). These results indicated that when the enzymes are immobilized closer to the nanotube, they are still accessible to substrate.

Conclusions

We have demonstrated a new approach for the general immobilization of enzymes on a surface using comb-branched DNA. Distances between an enzyme and the surface or between multiple enzymes can be controlled by modifying the comb-branched DNA, allowing greater design flexibility than current approaches. Amperometric concentration studies demonstrated both ADH and GDH were active upon attachment to the comb-branched DNA via amide bond formation. The location of the enzymes along the foundation strand and the length of the foundation strand affected the current response for the modified electrodes, but to different extents for each enzyme. While V_{max} decreased with foundation length for both enzymes, enzyme location on a specific foundation strand led to significant differences in V_{max} for ADH. In all cases, K_m was relatively unaffected. These proof-of-principle results indicate that while the immobilization approach is general, enzyme response can be tuned by simple changes to the comb-branch structure. With appropriate modifications to the method, such as changing the modifications on the branch strands for enzyme immobilization, multiple-enzyme attachments and more complex systems will be readily achieved.

Acknowledgments

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