

Electrochemical Sensing of the Peptide Drug Exendin-4 Using a Versatile Nucleic Acid Nanostructure

Niamat Khuda, Subramaniam Somasundaram, and Christopher J. Easley*



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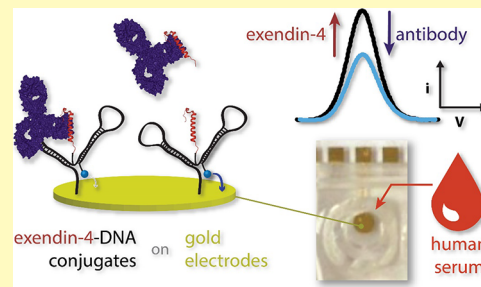
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Supporting Information

ABSTRACT: Although endogenous peptides and peptide-based therapeutics are both highly relevant to human health, there are few approaches for sensitive biosensing of this class of molecules with minimized workflow. In this work, we have further expanded on the generalizability of our recently developed DNA nanostructure architecture by applying it to electrochemical (EC) peptide quantification. While DNA-small molecule conjugates were used in a prior work to make sensors for small molecule and protein analytes, here DNA-peptide conjugates were incorporated into the nanostructure at the electrode surfaces, and antibody displacement permitted rapid peptide sensing. Interestingly, multivalent DNA-peptide conjugates were found to be detrimental to the assay readout, yet these effects could be minimized by solution-phase bioconjugation. The final biosensor was validated for quantifying exendin-4 (4.2 kDa)—a human glucagon-like peptide-1 receptor agonist important in diabetes therapy—for the first time using EC methods with minimal workflow. The sensor was functional in 98% human serum, and the low nanomolar assay range lies between the injected dose concentration and the therapeutic range, boding well for future applications in therapeutic drug monitoring.

KEYWORDS: biosensor, human blood, peptide, exendin-4, diabetes, electrochemistry, square-wave voltammetry, immunoassay



Peptides play significant roles as selective and efficient signaling molecules by binding to cell surface receptors and triggering intracellular effects.¹ The use of peptides as therapeutic agents has increased recently, owing to their high potency, selectivity, tolerability, and safety profiles in humans.^{2–4} For diagnostics, it is also important to monitor secretion of endogenous peptides such as hormones and cytokines.^{5–7} For such therapeutic and bioanalytical applications, it is essential to have a sensitive, specific, and accurate method for peptide quantification. To date, various standard methods have been developed for peptide quantification, such as chromatographic assays (LC–MS/MS), electrophoretic immunoassays, radioimmunoassays (RIA), and enzyme linked immunosorbent assays (ELISA).^{8–12} However, complex instrumentation and high cost limit the application of chromatographic or electrophoretic methods in routine analysis. While ELISA is the classical analytical approach due to its high sensitivity and throughput, repeatability is sometimes an issue,¹³ and the ELISA workflow is prohibitive for many applications.¹⁴ RIA has similar issues with hazardous, radioactively-labeled antigens. Moreover, these methods are expensive, time consuming, and require professional personnel, hindering their translation to point-of-care assays.

Diabetes is a complex endocrine metabolic disease, considered to be a major global health care problem.¹⁵ The disease arises due to insulin deficiency (type 1 diabetes) or insulin resistance (type 2 diabetes) and is associated with impaired secretion of peptides from islets.¹⁶ Exendin-4 is a

human glucagon-like peptide-1 receptor (GLP-1R) agonist with a molecular weight of 4.2 kDa, originally isolated from the venom of *Heloderma suspectum* (Gila monster).¹⁷ Exenatide—a synthetic version of exendin-4 approved by the U.S. Food and Drug Administration in 2005 for adjunctive treatment of type 2 diabetes¹⁸—regulates glucose metabolism and insulin secretion by enhancing glucose-dependent insulin secretion and suppressing elevated postprandial glucagon secretion.¹⁹ Due to the narrow therapeutic range and the varying nature of blood glucose levels, improved methods that allow regular monitoring of blood concentrations of exendin-4 are needed to achieve sustained efficacy in patients. Thus, the development of an amperometric or voltammetric electrochemical (EC) biosensor for exendin-4 quantification could make an immediate impact on human health monitoring.

Other groups have developed DNA-based sensors for quantifying protein-ligand binding based on chemically modified DNA strands that are bound to electrodes via DNA hybridization.^{20,21} In our recent work, we reported a highly sensitive and versatile DNA nanostructure probe for

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quantifying a range of analytes from small molecules to large antibodies.²² Compared to other work, this nanostructure sensor has the advantage of being inexpensive and modular, it consists of a single modified DNA molecule covalently attached to the electrode surface (which should improve sensor stability over time), and it has the potential to serve as a consistent scaffold for sensing a wider range of analytes—from small molecules through large proteins. In principle, this new platform is also suitable for sensing any peptide that can be conjugated to DNA and has a large binding partner, yet experimental proof has not been demonstrated to date. In this manuscript, we devise a method to apply our DNA nanostructure sensor architecture²² to quantify the peptide drug, exendin-4, which has not previously been measured using voltammetric or amperometric electrochemical readout, and we show that the sensor is functional in 98% human serum.

MATERIALS AND METHODS

Reagents and Materials. All solutions were prepared with deionized, ultra-filtered water (Fisher Scientific). Reagents 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), magnesium chloride hexahydrate from OmniPur, and sodium chloride were purchased from BDH. Bovine serum albumin (BSA) was obtained from VWR. Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), mercaptohexanol (MCH), gold etchant, and chromium etchant were from Sigma-Aldrich. Gold-sputtered on glass (GoG) (5 nm Cr adhesion layer, 100 nm Au layer) was purchased from Deposition Research Lab, Inc. (St. Charles, MO) with dimensions 25.4 mm × 76.2 mm × 1.1 mm. AZ 40XT (positive thick photoresist) and AZ 300 MIF developer were from Microchemicals, polydimethylsiloxane (PDMS) from Dow Corning, and dimethyl sulfoxide (DMSO) from Anachemia. Exendin-4 was purchased from LifeTein, monoclonal Anti-Exendin-4 antibody from Abcam (ab23407), and protein-oligo conjugation kits from Solulink. Custom, methylene blue-conjugated DNA (MB-DNA) was purchased from Biosearch Technologies (Novato, CA), purified by RP-HPLC. Thiolated DNA was obtained from Integrated DNA Technologies (IDT; Coralville, Iowa). T4 DNA ligase (400,000 units) and adenosine triphosphate (ATP, 10 mM) were from New England Biolabs. Prescreened human serum samples were acquired from BioIVT. Sequences of DNAs used in this work are listed in Table S1. “Measurement buffer” consisted of 10 mM HEPES at pH 7, 0.5 M NaCl, and 0.1% BSA.

EXPERIMENTAL METHODS

On-Electrode Surface Bioconjugation. Protein-oligo conjugation kits (Solulink) were used to activate exendin-4 for bioconjugation by modifying with S-HyNic (Solulink reagent), and anchor-DNA was modified with a 4FB cross-linker (Solulink reagent) to activate the amine. HyNic-modified exendin-4 was diluted to a final concentration of 8 μ M with 1× conjugation buffer and 10× TurboLink catalyst buffer (final catalyst buffer concentration was 1×). The DNA nanostructure was constructed via enzymatic ligation on the electrode surface as before²² (details below) using 100 nM of S-4FB-modified anchor-DNA. Later, 20 μ L of 8 μ M HyNic-modified exendin-4 was introduced into each electrochemical cell and incubated for 3 h at room temperature. The electrode was then rinsed and incubated with measurement buffer for at least 30 min prior to sensing.

Solution Bioconjugation. Peptide-oligo conjugates were alternatively prepared in free solution (referred to as “free solution conjugation”) using Protein-oligo conjugation kits (Solulink) by mixing 1.5 μ L of 10 μ M S-4FB-modified anchor-DNA, 2 μ L of 160 μ M HyNic-modified exendin-4, 4.5 μ L of 1× conjugation buffer, and 2 μ L of 10× TurboLink catalyst buffer. The mixture was incubated overnight at 4 °C. Later, the DNA nanostructure was constructed via enzymatic ligation on the electrode surface as before²² (below) using 100 nM exendin-4-conjugated anchor-DNA.

DNA Nanostructure Synthesis by on-Electrode Enzymatic Ligation. Once the electrode was ready, a mixture containing 100 nM anchor-DNA (either S-4FB-modified or exendin-4-conjugated, from above), 100 nM MB-DNA, 1.0 mM ATP, and 1.0 μ L of 400,000 U T4 DNA ligase was prepared in ligation buffer (10 mM HEPES with 10 mM MgCl₂ at pH 7). A total of 100 μ L of this mixture was introduced into the electrochemical cell, and then the cell was wrapped in parafilm and incubated for 6 h at room temperature. The electrode was rinsed with deionized water to remove enzymes and excess DNA, and then the cell was incubated with measurement buffer for at least 30 min prior to measurements.

Electrochemical Measurements. Electrochemical measurements were performed using a Gamry Reference 600 potentiostat. Once the working electrode was ready, the silver/silver chloride (3 M KCl) reference electrode (BASi) and platinum counter electrode (CH instruments) were introduced into the cell. Square-wave voltammograms were measured from −0.425 to 0 V (versus reference) with a step size of 1 mV, pulse height of 25 mV, and frequency of 100 Hz. Custom EC cells with 18 gold-on-glass (GoG) electrodes were used for sensing (see SI and Figure S1).

Anti-Exendin-4 Antibody Detection. DNA nanostructures with exendin-4-DNA conjugate as the anchor recognition unit were used for direct anti-exendin-4 (antibody) sensing via SWV in measurement buffer. The EC cell was emptied, and 20 μ L of anti-exendin-4 was introduced into the cell and incubated for 1 h. The sample was removed, and a final measurement was done in 100 μ L of the same buffer.

Exendin-4 Peptide Drug Quantification. DNA nanostructures with exendin-4-DNA conjugate as the anchor recognition unit were used for indirect exendin-4 sensing via SWV in measurement buffer. In a two-step process, 10 nM anti-exendin-4 (antibody) was pre-incubated with exendin-4 analyte (varying concentrations) in 100 μ L at 37 °C for 30 min, and the mixture was added to the sensor. To study kinetics, SWV was conducted every 3 min for a total of 75 min.

Measurements in Human Serum. Small volumes of exendin-4 and anti-exendin-4 antibody were spiked into human serum (98% serum with analyte). For the negative control (serum without exendin-4) only anti-exendin-4 antibody was spiked (99% serum). All samples were incubated at 37 °C for 30 min. After a baseline measurement in buffer, each 100 μ L mixture was added to the EC cell and incubated for 60 min. Finally, the serum mixture was removed, buffer was introduced, and a final measurement was done. SWV parameters were described above.

RESULTS AND DISCUSSION

Peptide Sensing Concept. In our previous work, a DNA-based assembly was enzymatically constructed directly on the electrode surface, resulting in a versatile electrochemical biosensor system.²² The nanostructure—a single DNA molecule that includes a thiol linkage to the gold electrode, an electrochemical label (MB), and an anchor binding moiety—serves as a stable scaffold with the binding and signaling labels held close to each other and at a fixed distance from the electrode surface. Our current understanding suggests that the nanostructure undergoes a change in effective mass upon target binding, likely with some added steric hindrance of movement, both of which will alter tethered-diffusion²³ of the MB label and cause a signal change at specific SWV frequencies.

In this work, the novel DNA nanostructure platform is applied for quantification of exendin-4, validating its use for peptide sensing. The peptide itself serves as the anchor-binding moiety in the anchor-DNA strand, after synthesis of novel exendin-4-nanostructure conjugates (peptide-DNA conjugates). As shown by the schematic in Figure 1, initially (left) the exendin-4-modified DNA nanostructure exhibited faster tethered diffusion (higher SWV peak current), which was then

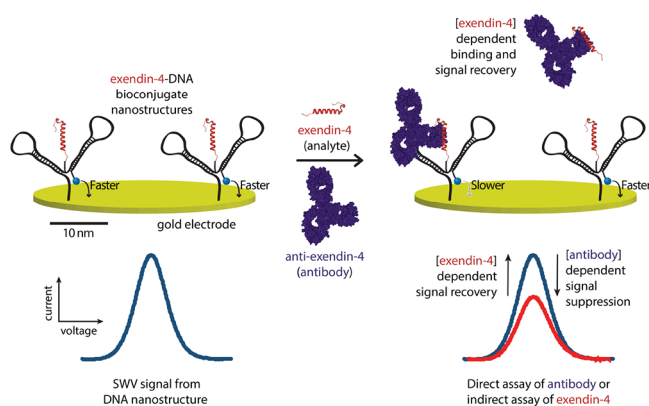


Figure 1. Faster diffusion of exendin-4-nanostructure conjugates is hindered by anti-exendin-4 (antibody) binding, and SWV current change is proportional to antibody concentration. For the peptide assay format, this effect can be blocked in a competitive assay workflow, and the SWV current recovery is proportional to exendin-4 concentration.

slowed by binding with anti-exendin-4 (antibodies), causing SWV signal suppression (right). This effect can be blocked through a competitive assay workflow, where pre-incubation of the antibodies with exendin-4 (analyte) in solution leads to reduction in concentration of the antibody-nanostructure complex on the surface, and signal recovery is observed in proportion to the analyte concentration.

Peptide-Oligo Bioconjugation. Commercial solid-phase synthesis, the conventional method for many oligonucleotide bioconjugates, is expensive and can result in very low yields. For instance, in our recent work²² the cost for tacrolimus-modified DNA anchor was found to be 4950 USD for 10.29 nmol, a rate of 481 USD nmol⁻¹. For peptide-DNA conjugates, syntheses must be customized, increasing costs further. Instead of this expensive route, we used commercial kits with some minor protocol changes. Exendin-4 was attached to an amine-modified anchor-DNA using a protein-DNA conjugation kit (Solulink), with three simple steps (Figure 2A). Exendin-4 was activated for bioconjugation by modifying with S-HyNic (generating “activated extending-4”), and anchor-DNA was modified with a 4FB cross-linker to activate the amine. The reaction of the two activated biomolecules resulted in the formation of a conjugate bond (exendin-4-DNA) stable up to 92 °C and over a range of pH from 2.0 to 10.0. One major advantage of this method is the mild reaction conditions that do not require metals or redox reagents, and thus bioconjugation should not affect the ability of exendin-4-DNA to bind antibodies for EC detection. This technology can feasibly be employed to append a variety of peptides, or antibody-binding epitopes of any protein, with the anchor DNA to allow quantification of the relevant analyte or to promote assay multiplexing.

Surface Bioconjugation. We initially performed bioconjugation directly on the electrode surface onto already-formed nanostructures, allowing convenient washing of unused reagents to avoid affinity purification.²² First, thio-DNA was immobilized on the gold electrode in a self-assembled monolayer, and then anchor-DNA and MB-DNA were introduced and enzymatically ligated on the electrode. After construction, unreacted strands and enzymes were washed away from surface-immobilized nanostructures. The SWV current was measured as a blank, and then 20 μ L of activated

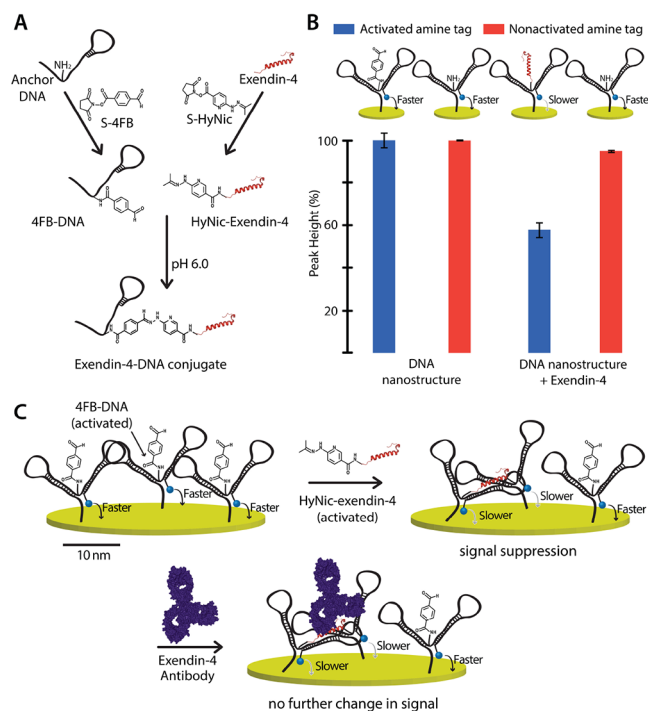


Figure 2. (A) Exendin-4-DNA bioconjugation, nominally at the N-terminus of the peptide. (B) Activated amine tagged nanostructure showing a significant SWV signal suppression (blue bars) compared to a non-activated nanostructure (red bars), confirming peptide-oligo bioconjugation. Error bars report standard deviations of triplicate electrode preparations. (C) Multivalent surface conjugation may result from the N-terminus and multiple lysine or arginine residues in the peptide sequence, masking the antibody-induced changes in signal.

exendin-4 solution was incubated on the electrode for bioconjugation. The electrode was rinsed to remove unreacted exendin-4, and the current was measured again. Figure 2B depicts the signal response of exendin-4 modified and unmodified nanostructures. In the absence of activated amine, formation of a conjugate bond between the anchor-DNA and exendin-4 could not take place, and only a 6% suppression of the initial signal was observed (red bars). In contrast, we observed a 42% drop in the peak height after conjugation with activated amine (blue bars), indicating that the tethered diffusion of the redox molecule was slowed by exendin-4 attachment, confirming successful bioconjugation onto the electrode surface.

However, follow-up studies showed that these exendin-4 modified nanostructures—made by surface conjugation—exhibited only a slight change in response to exendin-4 antibody addition. Our hypothesis for this lack of signal change is based on the multivalency of the surface (Figure 2C). Ideally, bioconjugation takes place at the N-terminal residues on the peptide, making 1:1 peptide-nanostructure conjugates at electrode surfaces that have predictable surface structures. However, when peptides comprise lysine or arginine residues (amines), it is possible that on-electrode reactions generate multivalent bioconjugates, where the peptide is attached to multiple, closely-spaced DNA nanostructures (Figure 2C). Multivalency of surface conjugation would result in restricted nanostructures, showing a large decrease in current after conjugation, but it would blunt antibody-induced changes in signal during sensor tests, as observed. Indeed, exendin-4 has

two lysine residues (positions 12 and 27 of the 39-amino acid peptide) and one arginine residue (position 20). Therefore, multivalency of surface conjugation is feasible, and we hypothesized that this was the cause of the negligible sensor responsiveness to antibodies.

It should be noted that the nanostructure distribution represented in Figures 1 and 2 were intentionally depicted to-scale, to our best approximation, which is why scale bars were included. Molecular sizes and lengths for DNA, peptides, and antibodies were based on structural data from The Protein Data Bank (PDB IDs: 5DK3, 1JRJ),^{24,25} and nanostructure density on the electrode surface was based on our measurement of the number of moles of nanostructure, N_{tot} using the methodology described previously.^{26,27} The average distance between nanostructures was measured to be 27 nm (see SI), and of course, a range of distances around this value would be expected. These structural data support our hypothesis as in Figure 2C. Further, because multivalency could significantly enhance the reaction rate of the bioconjugation, it is likely that these multivalent conjugates would be more prevalent than the monovalent counterparts. Lastly, the hypothesis was supported by the fact that solution-phase bioconjugation resolved the issue, as discussed below.

Free Solution Bioconjugation Approach. To minimize effects of multivalency, we modified the protocol to a free solution conjugation with excess peptide, followed by enzymatic ligation at the surface. After this change, the final sensor was functional, exhibiting a 54.5% reduction in the SWV current when the exendin-4 antibody (100 nM) was added to the electrode. Considered together, these results suggest that when using the DNA nanostructure sensing platform for peptide or antibody sensing, the peptide sequence should be carefully considered. Surface bioconjugation should be useful for peptides without reactive amines in the structure (other than the N-terminus), while free solution conjugation should be used if lysine or arginine residues are present.

An advantage of this method compared to prior approaches is that peptide-oligos can be made in-house, without requiring custom conjugations by commercial sources. Further, our technology does not require a purification step, since it is defined by a site-specific probe construction through the ligase enzyme followed by a surface hybridization and wash. This aspect is yet another significant advancement from our previous work, and the methods should position the sensor to be more accessible to others in the bioanalytical sector.

Antibody Induced EC Signal Suppression with DNA-Peptide Bioconjugate Nanostructures. After confirming antibody-induced decreases in tethered diffusion of the nanostructure, we expected this effect could be blocked by pre-incubating the antibodies with exendin-4 (analyte) followed by adding the mixture onto the electrode surface. In other words, we hypothesized that our nanostructure could be used for a competitive electrochemical immunoassay for exendin-4 quantification. The applicability of this sensing system will be limited by the pre-incubation antibody concentration; therefore, this value was optimized.

To obtain the optimal concentration of 10 nM, we titrated the nanostructure-modified electrode surface with anti-exendin-4 (antibody) (Figure S3). Figure S3A shows that titration with increasing concentrations of antibody suppressed signal monotonically, with effective saturation of the surface at 100 nM antibody. 10 nM was selected as the optimal concentration of exendin-4 antibody, both for the appreciable

signal drop (34%) within 10 min of incubation (Figure S-3B) and for the per assay cost savings.

EC Sensing of a Peptide Drug, Exendin-4. As noted earlier, monitoring of blood concentrations of this peptide drug is crucial for formulation optimization and suppression of diabetes symptoms in patients. To show proof-of-concept that our nanostructure sensor could be used for exendin-4 quantification, varied concentrations of exendin-4 were preincubated with antibody followed by dropping the mixture onto the sensor surface. Initially, a measurement was done every 3 min to evaluate binding kinetics. Figure 3A compares

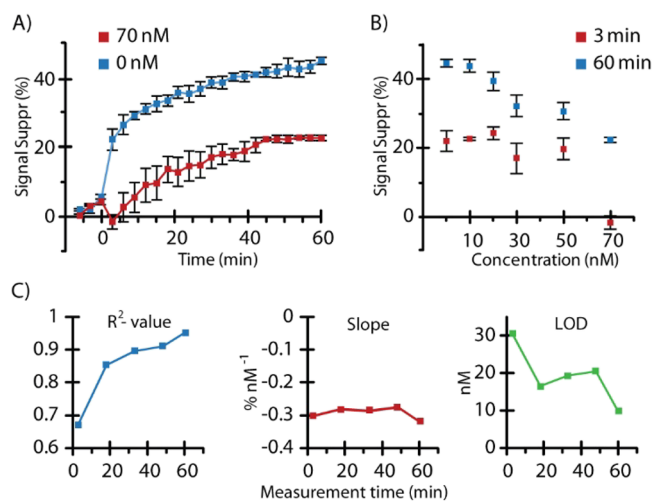


Figure 3. (A) Antibody to DNA-nanostructure binding kinetics were disrupted by the presence of the exendin-4 peptide analyte. (B) Calibration curves at 3 and 60 min. (C) Evaluation of the sensors response at five measurement times. Error bars in (A) and (B) report standard deviations using three different electrodes.

the relative differences in signal suppression in the presence of 0 or 70 nM exendin-4, where signal was suppressed faster in the absence of analyte and slower when exendin-4 competed with antibody binding to the DNA nanostructure. Figure 3B compares the calibration curves measured at 3 and 60 min after various concentrations were added. These results are consistent with a functional competitive immunoassay, as expected.

Figure 3C shows plots of the R^2 value (blue), magnitude of slope (red) of the linear calibration curve, and limit of detection, LOD (green) as a function of measurement time. These results indicate that with increasing measurement time, the R^2 and LOD values both improve, while sensitivity remains relatively constant. Considering the ease of use of the final sensor, we chose 1 h as an acceptable incubation time for the assay, and a final optimized calibration of this system was capable of exendin-4 quantification in the range of 10 to 70 nM, with a 3σ LOD of 6 nM (Figure 4A) and an R^2 of 0.985 for a linear fit (although the response is not expected to be precisely linear). To our knowledge, this DNA nanostructure sensor is the first example of a sensor capable of exendin-4 quantification using EC readout.

We finally investigated the stability of the sensor in undiluted human serum. Figure 4B demonstrates the signal suppression observed in spiked serum samples ($n = 3$). With exendin-4 (+), the percentage of signal suppression was blunted compared to controls with only antibody (−), agreeing with the results in buffer. Signal recoveries were determined to

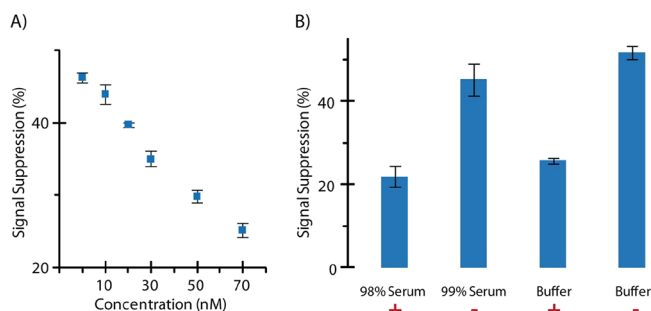


Figure 4. (A) Exendin-4 calibration curve showing good sensitivity in a range that overlaps with the therapeutic window. (B) A 70 nM exendin-4 (+) mixed with 10 nM antibody was spiked into undiluted serum and in buffer and compared to controls with only antibody (-). Expected trends for the competitive assay were observed. Three different electrodes were used for each of the three differently spiked serum samples and in the calibration experiments.

be high at 85% (+) and 88% (-). These results provide an important proof-of-concept that suggests our nanostructure sensor could function as a sensor of the peptide drug directly in human blood.

Clinical Relevance of Assay. Dosing of exendin-4 (i.e., exenatide) depends on the formulation. For example, a standard subcutaneous injection is dosed at $\sim 60 \mu\text{M}$, while an extended release formulation is dosed at $\sim 560 \mu\text{M}$.²⁸ The studied therapeutic and supratherapeutic levels in blood are 60 (250 pg. mL^{-1}) and 150 pM (630 pg. mL^{-1}), respectively. Although the dynamic range of our nanostructure sensor for exendin-4 (nM) does not yet reach the therapeutic range (pM), the sensors could be immediately useful for dosing and distribution studies at the injection site since the range is well below the injection concentration (μM). Another interesting application would be the direct detection of exendin-4 antibodies, since the peptide drug was shown to elicit immune responses with a high titer of anti-exendin-4 antibodies in 31.8% of patients.²⁸ Of course, more investigations will be necessary in this case, since various antibodies may be generated by the immune response, which have widely varying affinities toward the sensor surface. Because our sensors can detect either the antibody (direct assay) or the peptide (indirect assay), multiple applications are within reach after sensitivity improvements in the future.

CONCLUSIONS

In this work, we describe the development of an electrochemical biosensor system for the quantification of a novel peptide analyte for electrochemistry, exendin-4, which is a widely prescribed drug or diabetics. Exendin-4 was conjugated to the DNA nanostructure, and antibodies were shown to bind the conjugates and suppress SWV current. The sensing platform showed a limit of detection of 6 nM with a dynamic range of 10–70 nM, and the sensor was functional in 98% human serum. While multivalent DNA-peptide conjugates were found to be detrimental to sensor performance, solution-phase bioconjugation solved this issue.

More broadly, because our DNA nanostructure sensor architecture was used to detect small molecule drugs and antibodies in a prior work,²² this report has further expanded the generalizability of the platform into peptide sensing. Similar EC assays with minimal workflow could be developed for a variety of other peptides by merely changing the specific

peptide used in the bioconjugation step, providing opportunities in therapeutic and human health monitoring.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.1c02336>.

List of DNA sequences used, electrode preparation methods, electrochemical cell fabrication, DNA monolayer assembly, bioconjugation reaction details, and data analysis details (PDF)

AUTHOR INFORMATION

Corresponding Author

Christopher J. Easley – Department of Chemistry and Biochemistry, Auburn University, Auburn, Alabama 36849, United States; orcid.org/0000-0002-2403-4147; Email: chris.easley@auburn.edu

Authors

Niamat Khuda – Department of Chemistry and Biochemistry, Auburn University, Auburn, Alabama 36849, United States

Subramaniam Somasundaram – Department of Chemistry and Biochemistry, Auburn University, Auburn, Alabama 36849, United States; orcid.org/0000-0002-1064-4358

Complete contact information is available at: <https://pubs.acs.org/10.1021/acssensors.1c02336>

Author Contributions

N.K., S.S., and C.J.E. conceived the study, designed experiments, analyzed data, and collaboratively wrote the manuscript. N.K. and S.S. performed experiments under the supervision of C.J.E.

Notes

The authors declare the following competing financial interest(s): The assay system reported in this manuscript has been submitted for intellectual property protection; patent pending. The authors have entered into a licensing agreement with an interested party.

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