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An Electrochemical Aptamer-Based Sensor for Real-Time Monitoring of Insulin

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Abstract

In this manuscript, we developed an electrochemical, aptamer-based (E-AB) for the real-time monitoring of insulin. The sensor utilizes a redox label-modified guanine-rich aptamer which folds into a G-quadruplex for specific recognition of insulin. To develop a reproducible E-AB sensor employing insulin aptamer probes for the detection of insulin, 10% sodium dodecyl sulfate (SDS) pretreatment is crucial as it disrupts interstrand G-quartets. After sensor pretreatment with 10% SDS, more uniform sensor response is obtained. Upon introduction of insulin target, binding-induced steric hindrance quantitatively reduces the efficiency of electron transfer of a distal-end redox label leading to the rapid signal change within ~60 s. Testing demonstrates that the E-AB insulin exhibits a limit of detection of 20 nM and can be used to discriminate against both glucagon and somatostatin in Krebs–Ringer bicarbonate buffer, typically used in perfusion experiments. These results demonstrate that this assay has potential for rapid, specific, and quantitative analysis of insulin.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via on the ACS Publication website at DOI:***.

Structures of the E-AB insulin sensors, effect of 10% sodium dodecyl sulfate pretreatment on the Insulin-End sensor performance, square wave voltammetry current responses of the Insulin-End sensor vs. square root of the SWV frequencies, and effects of Redox Label Location on the E-AB insulin sensor Performance (Figure S1–S4)

Notes

The authors declare no competing financial interest.

Keywords

insulin; aptamers; sensors; G-quadruplex; electrochemistry

Detection of the polypeptide insulin is an important endeavor as it can potentially be a predictive marker of early onset type 2 diabetes.^{1–2} As such there have been numerous approaches reported in the literature for the detection of insulin including enzyme-linked immunosorbent assays or radioimmunoassays.³ While these methods offer clear advantages in the clinical setting in terms of sensitivity and specificity, there are some disadvantages like high instrument and operational costs, laborious manipulation, and long detection time. Aptamers offer a different mode of specific detection and have gained popularity as a result of easier synthesis protocols, lower cost, better stability, the ability to bind a broad range of target analytes, and more sustainable to repeated denaturation and renaturation.⁴ Given these properties, aptamers are considered as ideal recognition elements for biosensing and have been extensively adopted in numerous biosensors especially in electrochemical, aptamer-based (E-AB) sensors.^{5–8}

Recently, Lai and coworkers introduced a functional nucleic acid sensor for the detection of insulin that utilizes an insulin-like polymorphic region-based (ILPR) sequence of DNA as the sensing element.⁹ In their report, Lai and coworkers propose that an insulin-induced conformational change in the structure of the IPLR leads to electrochemical signal. This observation is supported by circular dichroism spectroscopic studies. While their sensor employed a naturally existing insulin binding sequence of deoxyribose nucleic acid (DNA), the sequence was not selected through SELEX (Systematic Evolution of Ligands by Exponential Enrichment).

In this manuscript, we developed an E-AB insulin sensor based on an *in vitro*-selected aptamer for sensitive, specific, selective, and real-time monitoring of insulin. To fabricate this sensor, we employed a redox label-modified guanine-rich aptamer, first selected and reported by Yoshida et al (termed IGA3),¹⁰ as a sensing element. As is typical in this class of sensor, the aptamer is immobilized on a gold electrode surface via formation of a self-assembled monolayer containing both thiolated aptamer and 6-mercapto-1-hexanol. The design and proposed sensing mechanism of the insulin sensor is shown in Scheme 1. Our studies indicate that the sensing mechanism of this sensor architecture is a result of insulin-binding-induced steric hindrance of the aptamer probe in contrast to a structure switch. The E-AB sensor is highly sensitive and selective, exhibiting a 20 nM limit of detection, and is functional in Krebs–Ringer bicarbonate (KRB) buffer, commonly used in islet perfusion experiments.¹¹ The sensor also demonstrates high specificity as it can discriminate between insulin and other closely related analogues due to subtle structural differences. Given these performance characteristics, we believe that our sensor offers utility in monitoring insulin from pancreatic islets.

EXPERIMENTAL SECTION

Chemicals and Aptamer Probes

6-mercapto-1-hexanol (C6-OH), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma (Tris) base), hydrogen chloride (HCl) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sulfuric acid (H₂SO₄), sodium bicarbonate (NaHCO₃), calcium chloride (CaCl₂), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), 10% sodium dodecyl sulfate (SDS), glucagon, somatostatin, bovine albumin serum (BSA), insulin solution from bovine pancreas were used as received (Sigma-Aldrich, St. Louis, MO). All other chemicals were analytical grade. All of the solutions were prepared using ultrapure deionized (DI) water purified with a Biopak Polisher (18.2 MΩ•cm, Millipore, Billerica, MA). The interrogation buffer was Tris buffer (50 mM tris, 10 mM KCl, 100 mM NaCl, 50 mM MgCl₂, pH 8.0). Another interrogation buffer used was KRB buffer (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 0.1% BSA, pH 7.4).

The insulin aptamer probes synthesized by Biosearch Technologies, Inc. (Novato, CA) were used as received. A C6-disulfide (HO-(CH₂)₆-S-S-(CH₂)₆-5'DNA) linker and a MB redox label was attached at the 5' and 3' terminus of both Insulin-End and Insulin-Middle probes, respectively (Supporting Information Figure S1). The Insulin-End probe: 5'-HS-C6-AAAAGGTGGTGGGGGGGGTTGGTAGGGTGTCTTCT-MB-3'; the Insulin-Middle probe: 5'-HS-C6-AAAAGGTGGTGGGGGGGGTTGGT(MB)AGGGTGTCTT C-3'.

Electrochemical, Aptamer-Based (E-AB) Insulin Sensor Fabrication

Before sensor fabrication, 2 mm diameter gold disk electrodes (CH Instruments, Austin, TX) were polished with 1 μm diamond slurry (Buehler, Lake Bluff, IL). To remove bound particulates, electrodes were sonicated in a low-power sonicator for ~5 min. Electrochemical cleaning was then performed after mechanical cleaning. A series of oxidation and reduction cycles in 0.5 M H₂SO₄ were performed. The amount of charge consumed during the reduction of the gold surface oxide in 0.05 M H₂SO₄ were used for calculating the real surface area of each electrode. According to a reported value of 400 μC cm⁻², the roughness factor was calculated based on the ratio between measured area and geometric area, and the factor of the electrodes used in this study ranged from 1.0–1.5.

Sensor fabrication includes several steps. First, to reduce the disulfide bonds, 1 μL of the 200 μM insulin aptamer probe solution was mixed with 1 μL of 10 mM TCEP at room temperature (~23 °C) for 1 hr. Second, the solution was diluted with a 50 mM Tris buffer with 10 mM KCl, 100 mM NaCl, 50 mM MgCl₂, pH 7.4. The diluted solution of the insulin aptamer probe (2 μM) was annealed at ~90 °C for 5 min and then chilled to ~4 °C gradually to obtain G-quadruplex conformation. After-wards, the insulin aptamer probe solution was further diluted to 0.05 μM with the Tris buffer, in which freshly cleaned gold electrodes were incubated overnight at ~4 °C. Third, the electrodes were passivated with 30 mM C6-OH for 5 hr at ~4 °C. We hypothesize that annealing is beneficiary for the formation of intrastrand G-quadruplex structural motifs and that the long passivation times helps forming a stable

self-assembled monolayer. The use of longer passivation times is not unprecedented, and several examples in the literature use this approach for improved stability.^{12–14–22}

After sensor fabrication, the fabricated sensors were placed in 10 % sodium dodecyl sulfate (SDS) for 15 min. After 20 s DI water rinse, the sensors were placed in Tris buffer or KRB buffer. To disrupt the interstrand G-quartets between the neighboring insulin aptamer probes, a chaotropic agent, 10% SDS was used. In order to obtain uniform probe orientation, 10% SDS pretreatment was demonstrated to be necessary (Supporting Information Figure S2). Without this pretreatment step, the sensors did not perform reproducibly.

To determine the density (Γ) of aptamer probes on the electrode surface, cyclic voltammetry (CV) peaks were integrated when using slow scan rates (e.g. 20, 50 and 100 mV s⁻¹) according to the following equation (eq1).

$$\Gamma = Q/nFA \quad (1)$$

In eq 1, Q is charge consumed/produced during the CV scan, n is the number of electrons transferred per equivalent ($n = 2$ for MB), F is the Faraday's constant, and A is electrode area. All reported Γ values reported represent the average value from voltammograms using the three aforementioned different scan rates.

Electrochemical Measurements

A CHI 630E Electrochemical Workstation (CH Instruments, Austin, TX) was used for electrochemical measurements at room temperature. We employed alternating current voltammetry (ACV), square wave voltammetry (SWV), and CV to interrogate the sensor surfaces. AC voltammograms (ACVs) were collected using frequencies between 5–300 Hz with an amplitude of 25 mV. Likewise, SWV voltammograms (SWVs) were collected using frequencies of 5–500 Hz with an amplitude of 25 mV. CV scans were recorded using scan rates between 0.02–0.1 V s⁻¹. All measurements were performed using a three-electrode system including a 2-mm diameter Au working electrode, a platinum wire counter electrode, and a Ag/AgCl (3 M KCl) electrode reference electrode (CH Instruments, Austin, TX).

Before introduction of insulin, ACVs of the sensors were recorded in Tris buffer, or KRB buffer. In most experiments, 20 μ M insulin was used to test sensor performance. The interval for ACVs collection was set as 10 min. For the dose–response curve performed in Tris buffer, the concentrations used were 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, and 20 μ M; for the dose-response curve performed in KRB buffer, the concentrations used were 200 nM, 1 μ M, 5 μ M, 10 μ M, and 20 μ M. The sensor was equilibrated for 30 min at each target concentration. The limit of detection (LOD) is determined based on a signal-to-noise ratio (S/N) of 3. The ACVs recorded in the absence of insulin for a period of 30 min was used to determine the “noise”. The % signal enhancement (SE) and % signal suppression (SS) (eqs 2 and 3) was calculated using the following relationships

$$\%SE = [(I - I_0)/I_0] * 100 \quad (2)$$

$$\%SS = [(I_0 - I)/I_0] * 100 \quad (3)$$

where I_0 is the baseline-subtracted peak current in the absence of insulin, and I is the baseline-subtracted peak current obtained in the presence of insulin.

RESULTS AND DISCUSSION

The electrochemical, aptamer-based sensors (E-AB) described here are based on an aptamer sequence previously published that has a high propensity for G-quadruplex formation. We investigated several sequence variants including the parent sequence (unaltered, with redox probe placed either at the distal end or internal to the sequence), and the modified parent sequence (altered, with 15-mer nucleotides extension for inducing a stem-loop structure). Presented below are data from the best performing sequence, the parent sequence with redox label at the distal end (termed Insulin-End). Representative data of the other sensors are provided in supporting information.

Sensor Surface Pretreatment for Improved Performance

We first characterized the E-AB Insulin-End sensors using ac voltammetry (ACV) and square wave voltammetry (SWV), and found that surface pretreatment is required for reproducible sensor performance. For example, while initial voltammetric interrogation provided a well-defined MB peak consistent with the standard reduction potential of MB in a pH 8.0 buffer (Tris) (Supporting Information Figure S2), the sensor performance to the addition of insulin was not consistent, as % signal suppression varied from 20% to 50% (data not shown). We hypothesized this irreproducibility was the consequence of the ability of neighboring DNA strands to form interstrand G-quadruplex conformations, which prevents formation of intrastrand G-quadruplex needed for insulin binding. While we cannot say, unequivocally, that aptamer packing heterogeneity and inter-strand interactions are the cause of the sensor-to-sensor variability, we observe marked changes in reproducibility in sensor performance after this pretreatment step as described below. Furthermore, literature suggests that the nature of the surface and the packing of nucleic acids could have consequential impact on sensor performance.^{15–16}

To mitigate sensor performance issues, and reduce interstrand interactions, we introduced a surface pretreatment step. Specifically, we treated sensors by incubating in a 10% SDS buffer solution. Typically, sensors treated with a 15 min 10% SDS incubation exhibited a signal increase ranging from 10% to 40% (Supporting Information Figure S2). Although we cannot rule out that disruption of intrastrand G-quadruplex formation in the individual aptamer strands might occur, the pretreatment results from 20 individual sensors shown in Figure S2 indicated that intrastrand G-quadruplex was more favorable induced once the sensor was interrogated in Tris buffer, as % signal change increases after 10% SDS incubation. Thus, we hypothesize that this signal increase is indicative of a change from potential interacting neighboring DNA strands to individual intrastrand G-quadruplex formation once the sensor was interrogated in Tris buffer after 10% SDS incubation.

(Scheme 1 – Top). Sensor-to-sensor variability is likely representative of aptamer packing heterogeneity.

With the optimal surface pretreatment protocol established, we found that the E-AB insulin sensor responds quantitatively to the presence of insulin and does so rapidly. Specifically, addition of 20 μM insulin resulted in $42.4\% \pm 0.9\%$ and $31.1\% \pm 2.2\%$ signal suppression using optimized (described below) ACV 200 Hz and SWV 500 Hz respectively (Figure 1). Furthermore, the response rate of the E-AB insulin sensor is rapid. Using ACV at 200 Hz, we find that 90% of the observed signal suppression is achieved within 60 s of target addition for the Insulin-End sensor (probe density $3.0 \pm 0.3 \times 10^{11}$ molecules cm^{-2}); The sensor equilibrates to the addition of target within 20 min (Figure 2).

Proposed Signaling Mechanism of E-AB Insulin Sensor

To better understand the signaling mechanism of the sensor, we evaluated the ACV frequency-dependent current of the sensors with and without the presence of 20 μM insulin (Figure 3). For the electrochemical sensors employed a redox moiety,^{25–27} the applied frequency is significant to the sensor response. For example, when the ACV frequency employed to interrogate the surface is slower than the electron transfer rate between MB and the electrode surface, the peak current increases. Above a threshold frequency, faster than that of the electron transfer rate, the MB peak current decreases. When using sensors fabricated with the Insulin-End aptamer, this threshold frequency is observed to be ~ 200 Hz. In the presence of insulin, the increase in the MB current was observed from 5 Hz with current plateauing at 75 Hz, and the declining frequencies >200 Hz. As can be seen, insulin binding induced significant signal suppression across ACV frequencies, nevertheless, ACV profiles for the absence and presence of insulin are similar, which indicates the proposed binding-induced steric hindrance mechanism.

In addition to ACV, we also employed SWV for monitoring changes in electron transfer rate of MB. SWV is commonly used to interrogate E-AB sensor surfaces. Changes in SWV frequency also offers the potential to tune the sensitivity of E-AB sensors and switch the signaling polarity of resulting sensor.^{20–22} SWV peak currents increase with increasing SWV frequency both with and without insulin (Figure 3). Both target-bound and-unbound states matched well with the SWV theory and the differential current is directly proportional to the square root of the SWV frequency (Supporting Information Figure S3).²³ The comparable SWV profiles for the bound and unbound states imply the proposed binding-induced steric hindrance mechanism.

In addition to providing information about the signaling mechanism, variation of voltammetric interrogation frequency represents a relatively facile means of optimizing sensor sensitivity (Figure 4). A plot of % signal suppression as function of the applied ACV and SWV frequencies (Figure 4) yields optimal signaling performance of $42.4 \pm 0.9\%$ signal suppression with ACV at a frequency of 200 Hz and a maximum of $31.1 \pm 2.2\%$ signal suppression using SWV at a frequency of 500 Hz. Since maximum % signal suppression was obtained at a ACV frequency of 200 Hz, all the other experiments in this study were performed at this frequency.

Sensor Characteristics—The E-AB insulin sensor responds quantitatively to the presences of insulin. Specifically, the dose-response curve, fit to a one site binding model, Langmuir-like isotherm yielded a monotonic signal change (suppression) with respect to insulin concentration (Figure 5). From this data, the limit of detection (LOD) of the sensor was determined according to $s/n = 3$ to be 20 nM, and the dynamic range of the sensor was in a range between LOD and saturation concentration, which is between 0.02 and ~5 μ M. Furthermore, the dissociation constant (K_d) was 240 ± 65.0 nM. These values are consistent with the shape of the dose-response curve.

To compare the LOD of the electrochemical Insulin-End sensor we developed with existing methods adopting the IGA3 aptamer as the sensing element, we listed the sensing mechanisms and LODs of recently developed insulin sensors^{10, 24–30} (Table 1). Comparing with all the other insulin sensors, the LOD of our sensor is equivalent, apart from the insulin sensor based on graphene field-effect transistor.³⁰

Sensor Specificity and Selectivity—The E-AB insulin sensor is specific to insulin. To test specificity, we challenged the sensor against two potential interference: glucagon and somatostatin. As insulin analogues, glucagon promotes glucose storage by liver and regulates the secretion of insulin. Likewise, somatostatin plays a role in regulating the secretion of insulin. Due to the physical roles glucagon and somatostatin displayed in blood glucose regulation, it is crucial to develop an insulin sensor that could be used to detect insulin in the presence of inferences such as glucagon and somatostatin. The E-AB insulin sensor was first exposed to Krebs–Ringer bicarbonate (KRB) buffer containing 0.1% bovine serum albumin (BSA), which is different from pure Tris. After sensor signal stabilized, insulin solutions of increasing concentrations were introduced in the KRB buffer (Figure 6). The LOD of the Insulin-End sensor in KRB buffer was determined to be 200 nM; the dynamic range of the sensor in KRB buffer was in a range between 0.2 and 20 μ M, and the K_d of the Insulin-End sensor in KRB buffer after fitting was determined to be 769 ± 171 nM. The K_d value of the Insulin-End sensor in KRB buffer is higher than the achievable K_d value 240 ± 65.0 nM in Tris buffer, nonetheless, the result indicates that binding of insulin to IGA3 ap-tamer in KRB containing BSA. Different from the achievable detection of insulin in KRB buffer, neither glucagon nor somatostatin addition, created any appreciable change in sensor signal between concentrations of 0.2–20 μ M (Figure 6). These results indicate that our electrochemical insulin sensor is potentially suitable for monitoring insulin secretion from pancreatic islet β cells as understanding the amount and temporal variation of insulin release from pancreatic islets of Langerhans is of high interest.³¹

Effects of Redox Label Placement on the Sensor Performance To design an E-AB sensor, an aptamer is usually adopted as the sensing element and a redox moiety is labelled on the aptamer sequence for generating detectable signal. While target-induced changes in the conformation of the aptamer probe is the core of the sensing mechanism of E-AB sensors, the readout is based on the reduction/oxidation current generated by the redox label. The placement of the redox probe can affect the signal attenuation obtained from the sensor. To test redox probe placement, we designed the Insulin-Middle sensor using the same IGA3 aptamer but with the MB label modified internal (the middle) to the probe aptamer. The procedure used for the Insulin-Middle sensor was based on the same experimental condition

optimized for the Insulin-End sensor. In the presence of 20 μM insulin, the Insulin-Middle sensor displayed 35.3 ± 1.9 % SS in comparison to 46.1 ± 4.1 % SS achieved for the Insulin-End sensor at an ACV frequency of 200 Hz (Supporting Information Figure S4). The higher % SS obtained from the Insulin-End sensor manifested that the Insulin-End sensor is a more advantageous insulin sensor design.

CONCLUSION

In this manuscript, we presented the first E-AB insulin sensor based on the guanine-rich IGA3 aptamer. The signaling mechanism for this sensor is proposed to be based on binding-induced steric hindrance of electron transfer between the redox label and electrode surface. We demonstrated that to achieve re-producible E-AB insulin sensors, 10% SDS pretreatment is invaluable as it prevents formation of interstrand G-quartets. The E-AB insulin sensor offers a LOD down to 20 nM and is rather rapid as 90% signal saturation was achieved within 60 s. Besides low LOD and fast kinetics, the E-AB insulin sensor is also sensitive, specific and selective, which is shown by the sharp slope of the calibration curve and its ability to discriminate against both glucagon and somatostatin in KRB buffer. Through assessing the effect of redox label placement on sensor performance, we validated that the Insulin-End sensor with a MB label modified at the proximal end of the IGA3 aptamer probe sequence is more advantageous than the counterpart with a MB label modification located at the middle of the IGA3 aptamer probe sequence. We believe this sensor can be used for real-time monitoring of insulin release in several *in vitro* settings..

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

E-AB	electrochemical, aptamer-based sensor
MB	methylene blue
SWV	square wave voltammetry
ACV	alternating current voltammetry
CV	cyclic voltammetry
SS	signal suppression
SE	signal enhancement

KRB	Krebs–Ringer bicarbonate buffer
LOD	limit of detection
K_d	dissociation constant

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Scheme 1.

Electrochemical, aptamer-based sensors for the detection of insulin rely on insulin-binding changes to the steric hindrance of signaling aptamer probes, resulting in a decrease in measured electrochemical signal. Prior to sensor testing, 10% SDS treatment was applied for disrupting the potentially formed interstrand G-quartets. When the sensors interrogate in tris buffer containing 10 mM KCl, the formation of both antiparallel and parallel G-quadruplexes is promoted.

Figure 1.

The E-AB sensor employing the insulin-end sequence exhibits a decrease in faradaic signal in the presence of insulin. (Left) 20 μM insulin resulted in $\sim 42\%$ SS at an ACV frequency of 200 Hz; (Right) 20 μM insulin resulted in $\sim 33\%$ SS an SWV frequency of 500 Hz.

Figure 2.

Kinetics of the E-AB Insulin-End sensor. Signal saturation was achieved within 60 s for the Insulin-End sensor in the presence of 20 nM and 20 μ M, and complete signal saturation was reached in 20 min. The kinetics was obtained at an ACV frequency of 200 Hz.

Figure 3.

Shown are ACV frequency-dependent current responses and SWV frequency-dependent current responses of the E-AB Insulin-End sensor before and after the addition of 20 μM insulin. ACV profiles are shown on the left; SWV profiles are shown on the right.

Figure 4.
% Signal Suppression of the E-AB Insulin-End sensor related to applied ACV and SWV frequencies. Maximum % SS was obtained at an ACV frequency of 200 Hz.

Figure 5.

Dose-response curve of the E-AB Insulin-End sensor in Tris buffer. The data were obtained from average of three different sensors (A). Shown on the right are ACVs of the sensor in the absence and presence of different concentrations of insulin (B). These data were collected at an ACV frequency of 200 Hz.

Figure 6.

Dose-response curves of the E-AB Insulin-End sensors for insulin, glucagon, somatostatin, respectively, in Krebs–Ringer bicarbonate buffer. The targets concentrations were, 200 nM, 1 μM, 5 μM, 10 μM and 20 μM. The data are averaged from three different sensors. These data were collected at an ACV frequency of 200 Hz.

Table 1.**Limit of Detection of Published Insulin Sensors**

Detection Methods	LODs	Refs
aptameric enzyme-inhibiting subunit for insulin sensing	1000 HM	10
fluorescence quenching based on CdSe/ZnS QDs@graphene oxide composite	2.74 nM	24
fluorescence quenching based on proximity of the fluorophore and quencher	9.97 nM	25
fluorescence quenching based on proximity of the fluorophore and graphene oxide	500 nM	26
colorimetric biosensing based on synergetic catalytic effect of Au-NPs and graphene oxide	10 nM	27
photoluminescence transduction based on target binding on aptamer-coated SWNTs	10 nM	28
peroxidase activity inhibition induced by competitive binding	1000 nM	29
aptameric nanosensor based on a graphene field-effect transistor	0.035 nM	30
E-AB insulin sensor based on binding-induced steric hindrance of electron transfer	20 nM	This work