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Nucleic Acid-based Electrochemical Sensors Facilitate the Study of DNA Binding by Platinum (II)-based Antineoplastics

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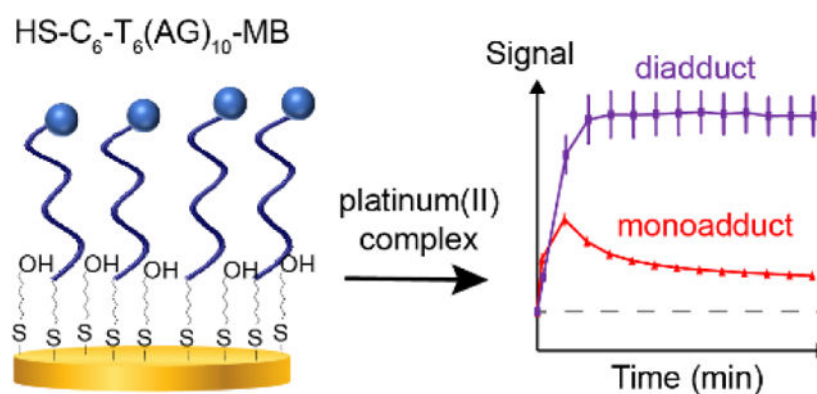
Abstract

DNA crosslinking agents such as cisplatin and related platinum(II) analogs are effective drugs to treat solid tumors. However, these therapeutics can cause high toxicity in the body, and tumors can develop resistance to them. To develop less toxic and more effective DNA crosslinkers, medicinal chemists have focused on tuning the ligands in square planar platinum(II) complexes to modulate their bioavailability, targeted cell penetration, and DNA binding rates. Unfortunately, linking in-vitro DNA binding capacity of DNA crosslinkers with their in-vivo efficacy has proven challenging. Here we report an electrochemical biosensor strategy that allows the study of platinum(II)-DNA binding in real time. Our biosensors contain a purine-rich deoxynucleotide sequence, T₆(AG)₁₀, modified with a 5' hexylthiol linker for easy self-assembly onto gold electrodes. The 3' terminus is functionalized with the redox reporter methylene blue. Electron transfer from methylene blue to the sensor is a function of platinum(II) compound concentration and reaction time. Using these biosensors, we resolve DNA binding mechanisms including monovalent and bivalent binding, as well as base stacking. Our approach can measure DNA binding kinetics in buffers and in 50% serum, offering a single-step, real-time approach to screen therapeutic compounds during drug development.

Nucleic acid-based sensors unveil the DNA binding mechanisms of platinum(II) complexes. Based on signals generated at dual frequencies of square wave voltammetry upon introduction of platinum(II) antineoplastics, the sensors can resolve formation of monovalent adducts and bivalent adducts on the purine-rich DNA sequences.

Graphical Abstract

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Keywords

DNA crosslinkers; platinum(II); cisplatin; biosensors; electron transfer

Introduction

Platinum-based chemotherapeutics are DNA-crosslinking agents that target rapidly dividing cancer cells by disrupting DNA synthesis.^[1] Since the approval of cisplatin by the Food and Drug Administration,^[2] cisplatin-based combination therapy remains one of the most effective treatments for solid tumors.^[3] Cisplatin's mechanism of action involves a cytosolic aquation process in which chloro ligands are displaced by water molecules to produce the DNA-binding mono-aqua and di-aqua forms. The aquated cisplatin can then enter the cell nucleus and react with the nucleophilic sites of purine bases, forming intra and interstrand crosslinks.^[4] Such crosslinks distort the three-dimensional structure of DNA^[5] and block binding of transcription factors, inducing apoptosis.^[6] Although effective in the treatment of solid tumors, cisplatin's side effects are considerable.^[7] Such side effects have motivated the development of new platinum-based antineoplastics aiming to improve therapeutic activity while also lowering toxicity.^[8]

The screening of cisplatin derivatives led to the discovery of carboplatin and oxaliplatin.^[9] In carboplatin, the stable cyclobutane-1,1-dicarboxylate (CBDCA) slows down DNA adduct formation kinetics in vivo,^[10] making the drug better tolerated by patients.^[11] Oxaliplatin contains a bulky diaminocyclohexane (DACH) ligand that shows strong antineoplastic activity against cisplatin-resistant tumors.^[12] Other non-classical platinum (II) complexes^[13] have been explored, and strategies to improve bioavailability via platinum (IV) prodrugs^[14] and nanodelivery^[15] have been successful to some extent. However, novel cisplatin derivatives targeting other cancer types remain elusive. This limitation is due in part because the measurement of platinum(II)-DNA adduct formation kinetics of lead compounds in vitro, which dictates structural parameters for in-vivo bioavailability, efficacy, and toxicity, remains challenging.

Preclinical evaluation of platinum(II)-DNA adduct formation kinetics is based on inductively coupled plasma-mass spectrometry (ICP-MS) to measure total ^{195}Pt levels in DNA samples.^[16] This technique features low detection limits and a high throughput, but requires

preparation of multiple reaction-terminated DNA-drug specimens sampled at specific time intervals,^[16] which is cumbersome. In addition, the obtained total ¹⁹⁵Pt levels post sample digestion do not report on all the kinetic processes involved in DNA bonding, making it hard to study the mechanisms of Pt-DNA adduct formation. Other instrument-based methods that exist for the determination of platinum(II)-DNA adducts (see Table S1) such as atomic absorption spectroscopy, NMR and optical tweezers^[16a, 16c, 17] are also based on sampled measurements and are, thus, not real time. As an alternative, nucleic acid-based electrochemical sensors (NBEs) – a technology that supports continuous monitoring of DNA-ligand binding interactions in physiological fluids^[18] – could fulfill the unmet need for real-time monitoring of adduct formation kinetics of cisplatin-like drugs in vitro.

Here we report a new sensor design that enables the study of DNA binding mechanisms of both classical (e.g., cisplatin, carboplatin, oxaliplatin) and nonclassical (e.g., phenanthriplatin) platinum (II)-based antineoplastics. Our strategy enables the continuous monitoring of adduct formation kinetics in real time in buffered solutions. The new sensor design achieves superior signaling output during Pt-DNA adduct formation than previous reports.^[19] The translational value of the new sensors is demonstrated via simultaneous monitoring of cisplatin and phenanthriplatin aqutation and adduct formation kinetics in buffered solutions over periods of days. Additionally, we demonstrate the detection of preaquated cisplatin and phenanthriplatin in 50% diluted serum. Our results indicate that NBEs could support rapid monitoring of platinum(II)-based therapeutics in clinical samples for dose scaling and therapy management applications. Thus, we postulate our NBEs could be used to estimate the chemotherapeutic potential of new cisplatin-like drugs, or as therapeutic drug monitoring platforms.

Results and Discussion

A cisplatin sensor design previously reported by Wu and Lai used the sequence HS–C₆–PO₄–AGAGAG–PO₄–MB, here called (AG)₃ (see Table 1).^[19] This sequence was selected because cisplatin ssDNA platination predominantly occurs at G sites.^[4a] In addition, by virtue of being a linear sequence, (AG)₃ achieved higher density packing of oligonucleotides on electrode surfaces relative to, for example, G-quartet forming sequences (e.g., GGGGGG), thereby increasing sensor signaling output. Using the (AG)₃ probe, Wu and Lai achieved ~120% signal-ON gain via AC voltammetry in the presence of 100 μM preaquated cisplatin. In contrast, here we enable the monitoring of cisplatin-DNA adduct formation kinetics by implementing two modifications to the original (AG)₃ sequence. First, we added six deoxythymidines at the 5' terminus of the sequence to increase the flexibility of the probe.^[20] And second, to increase the number of platination sites, we increased the number of AG motifs to ten (sequence T₆(AG)₁₀ in Table 1). Finally, we interrogated our sensors via square wave voltammetry which, unlike AC voltammetry, does not require parameter fitting to model circuits to explain experimental results.

When challenged with preaquated cisplatin, *cis*-[Pt(NH₂)₂(OH)₂] (see Methods), sensors functionalized with the sequence T₆(AG)₁₀ significantly outperformed sensors functionalized with (AG)₃ in signaling gain (Figure 1). To demonstrate this, we fabricated sensors by first depositing either the (AG)₃ or T₆(AG)₁₀ thiolated oligonucleotides at

a concentration of 2 μM from phosphate-buffered saline buffer containing 100 mM MgCl_2 , and then backfilling with a 30 mM solution of the blocking monolayer of 6-mercaptohexanol. The oligonucleotide packing densities for each set of sensors were $\Gamma_{(\text{AG})_3} = 21 \pm 2 \text{ pmol}\cdot\text{cm}^{-2}$ and $\Gamma_{\text{T}_6(\text{AG})_{10}} = 4.1 \pm 0.6 \text{ pmol}\cdot\text{cm}^{-2}$ (Figure S1). We then serially interrogated the sensors every 10 min via square wave voltammetry in HEPES buffer at pH = 5 (50 mM HEPES, containing 100 mM NaClO_4). We selected square wave frequencies of 300 Hz and 5 Hz because such frequencies achieved optimal sensor signaling outputs, reflect low- and high-frequency regimes in sensor frequency maps (Figure S2), and span experimental time constants across two orders of magnitude. DNA adduct formation was triggered by spiking *cis*-[Pt(NH₂)₂(OH)₂] to a final concentration of 100 μM (Figure 1A, B). Under these conditions, the spiked *cis*-[Pt(NH₂)₂(OH)₂] protonated into the reactive *cis*-[Pt(NH₂)₂(OH₂)₂]²⁺, which binds purines. At 300 Hz and $t \sim 120$ min after the spike, the $\text{T}_6(\text{AG})_{10}$ -functionalized sensors achieved a gain of $\sim 500\%$ vs only $\sim 8\%$ for $(\text{AG})_3$ -functionalized sensors. At 5 Hz, the $\text{T}_6(\text{AG})_{10}$ sensors achieved a gain of $\sim 100\%$ vs $\sim -50\%$ for $(\text{AG})_3$ -sensors.

Based on the kinetic data shown in Figure 1B, cisplatin binding to $\text{T}_6(\text{AG})_{10}$ sensors may follow a two-step process (Figure 1G). First, the activated *cis*-[Pt(NH₂)₂(OH₂)₂]²⁺ binds the oligos to form monovalent adducts (the rate limiting step),^[16c] physically stretching a population of oligos which, as a result, pushes the redox reporters away from the electrode surface, slowing electron transfer (Figure 1G, center panel). Monovalent adduct formation is observed in the first 0–12 min signal rise, with signaling going from 0% to $\sim 239\%$ at 5 Hz. The time constant of electron transfer for this oligo population matches the interrogation frequency of 5 Hz (red trace in Figure 1B). However, bivalent adduct formation has a lower kinetic barrier after the monovalent adduct is formed. Therefore, after the formation of the first bond the population of monovalent adducts quickly decreases, causing a decay in the 5 Hz sensorgram and progressively increasing sensor gain throughout the course of the experiment to saturate at $G_{300\text{Hz}} \sim 500\%$ after $t \sim 30$ min (purple trace in Figure 1B) at a frequency of 300 Hz. Measurements at the high frequency of 300 Hz match the time constant of electron transfer for a folded population of bivalent adducts (Figure 1G, right panel). We note that both mono and bivalent adducts can remain on the sensor surface (i.e., not all monovalent adducts convert to bivalent adducts), their relative populations reflected by the plateau signal observed at 300 Hz and 5 Hz, respectively.

To further highlight the importance of the poly-thymidine spacer, we performed similar measurements on a positive control sequence containing six deoxythymidines preceding the $(\text{AG})_3$ motif ($\text{T}_6(\text{AG})_3$ in Table 1, $\Gamma_{\text{T}_6(\text{AG})_3} = 17 \pm 1 \text{ pmol}\cdot\text{cm}^{-2}$), observing a ~ 12 -fold higher gain relative to the $(\text{AG})_3$ sensors at 300 Hz (Figure 1C vs A). As a negative control for the T_6 linker, we functionalized sensors with an $(\text{AG})_{13}$ sequence ($\Gamma_{(\text{AG})_{13}} = 1.0 \pm 0.3 \text{ pmol}\cdot\text{cm}^{-2}$), which had an equal number of nucleotides as $\text{T}_6(\text{AG})_{10}$ but devoid of the poly-deoxythymidine sequence. The resulting sensors showed a ~ 2.5 -fold lower gain at 300 Hz relative to those functionalized with $\text{T}_6(\text{AG})_{10}$ (Figure 1D vs B). Finally, sequences of equal length as $\text{T}_6(\text{AG})_{10}$ but with less AG motifs ($\text{T}_6(\text{AG})_3\text{T}_8(\text{AG})_3$, $\Gamma_{\text{T}_6(\text{AG})_3\text{T}_8(\text{AG})_3} = 6.3 \pm 0.9 \text{ pmol}\cdot\text{cm}^{-2}$) also showed a ~ 2.5 -fold lower gain at 300 Hz than $\text{T}_6(\text{AG})_{10}$ sensors (Figure 1E vs B).

Distinct from the other five sensors (Figure 1A – E), the (TA)₁₃ sensors are composed of sequences containing no G sites (sequence (TA)₁₃ in Table 1, $I_{(TA)13} = 4.4 \pm 0.1 \text{ pmol} \cdot \text{cm}^{-2}$, Figure 1F). Although it is well known that cisplatin predominately binds purine bases, Wenjuan Zeng and co-workers^[21] have demonstrated cisplatin can also bind pyrimidine bases with a lower yield. Thus, it is possible that the transient signal increase at 300 Hz and the sustained signal decrease at 5 Hz seen for (TA)₁₃ sensors (Figure 1F) is due to the same mechanisms of monovalent adduct formation–induced stretching followed by transient folding, but to a lesser extent. At 5 Hz, only sensors functionalized with T₆(AG)₁₀ produced positive signal gains, highlighting the importance of the T₆ linker and the intermediate (AG)₄ motif (compared to T₆(AG)₃T₈(AG)₃) to increase the sensitivity towards monovalent bond formation. Given these results, we moved forward with sequence T₆(AG)₁₀ for all subsequent measurements.

We next studied the effect of challenging T₆(AG)₁₀ NBE sensors with phenanthriplatin (Figure 2), a non-classical platinum (II)-based therapeutic that can only form monovalent adducts with DNA. Phenanthriplatin, (*cis*-[Pt(NH₃)₂-(phenanthridine)Cl]⁺), retains the square planar configuration of cisplatin. In a similar fashion as with *cis*-[Pt(NH₂)₂(OH)₂], NBEs functionalized with the T₆(AG)₁₀ sequence that were exposed to preaquated phenanthriplatin, *cis*-[Pt(NH₃)₂-(phen)(OH)]⁺, achieved the largest gains at both frequencies, 300 Hz and 5 Hz, relative to all other sequence controls (Figure 2A vs Figure S3A–E). However, a different binding mechanism is observed in this case. At short exposure times, $t < 3 \text{ min}$, the sensorgrams quickly raised to a maximum gain of $G_{300\text{Hz}} \sim 340\%$ and $G_{5\text{Hz}} \sim 300\%$. This increase in sensor signal was immediately followed by first-order signal decays (Figure S4, after at least 3 min). We propose that such observations reflect an initial stretching of one population of oligos following monovalent adduct formation (as seen in Figure 1), simultaneously coupled to stacking of the phenanthridine ligand to a second population that remains folded (Figure 2B).

The effect of the initial structural stretch is an increase in the population of oligos undergoing slow electron transfer, seen at $t < 3 \text{ min}$ in Figure 2A (red trace at 5 Hz). Ligand stacking can simultaneously trap a second population of oligos in a folded state able to transfer electrons faster, causing the signal increase seen at 300 Hz in the first 3 min (purple trace). However, both the oligo stretching and the stacking interaction can be reversed (Figure 2B, right), causing the signal to decay exponentially at $t > 3 \text{ min}$. As a reference, we note that base stacking is a structural property of ssDNA (in addition to dsDNA) and is expected in purine-rich sequences.^[22] We speculate that phenanthridine, given its planar structure, can stack with the purines in T₆(AG)₁₀.

To evaluate the hypothesis that phenanthridine (the ligand alone) can stack between the T₆(AG)₁₀ nucleotides, we challenged the sensors with 100 μM of this ligand and observed positive signal gains at both frequencies, $G_{300\text{Hz}} \sim 33\%$ and $G_{5\text{Hz}} \sim 33\%$ (Figure S5A). To confirm that the stacking interaction can be reversed and specifically generates the sensor response at 300 Hz, we repeated the experiment shown in Figure 2A but moved the sensors into phenanthriplatin-free buffered solution after 20 min (Figure 2C). Doing so we observed a 200% decay in sensor gain at 300 Hz, while the sensorgrams measured at 5 Hz (reflecting monovalent adduct formation) were less affected by the buffer wash.

These results confirmed that the stacking interaction affected the electron transfer measured at 300 Hz and is reversible (Figure 2D). Similar wash controls performed using *cis*-[Pt(NH₂)₂(OH)₂], which cannot intercalate, generated minimal changes in sensor signaling (Figure S5B), confirming that DNA binding mechanisms between these two therapeutics are different.

To further validate the generalizability of our NBE approach to the study of Pt(II)-DNA binding kinetics, we employed our T₆(AG)₁₀ sensors to monitor adduct formation with carboplatin and oxaliplatin (Figure 3). By virtue of having a worse leaving group (the cyclobutane dicarboxylate), carboplatin undergoes slower monovalent adduct formation kinetics relative to cisplatin.^[17c] However, the DNA binding mechanism is the same. As shown in Figure 3A, NBE sensors containing the T₆(AG)₁₀ probes showed monovalent adduct formation kinetics (at 5 Hz) that plateaued at *t* ~ 60 min, and slow bivalent adduct formation after that period. In contrast, the same sensors challenged with cisplatin peaked in the 5 Hz sensorgram at *t* ~ 12 min (Figure 3A vs Figure 1B, red traces).

Compared against the bivalent adducts formed by cisplatin in the first 30 min post drug challenge, the 300 Hz carboplatin sensorgrams never saturated (Figure 3A vs Figure 1B, purple traces). The slower binding kinetics of carboplatin relative to cisplatin, and the slower bivalent adduct formation rate relative to monovalent adduct formation have been previously measured by accelerator mass spectrometry,^[17c] validating the observations made with our sensors.

In the case of oxaliplatin, which contains one non-labile 1,2-cyclohexanediamine ligand and an oxalate leaving group, we observed a much lower platination density, with a net gain of *G*_{300Hz} ~ 50% at *t* ~ 120 min (ten-fold less than with cisplatin). This low gain indicates the bulkier nature of the cyclic ligand drastically decreases the density of platination in our sensors. Although the monovalent adduct formation kinetics we measured at 5 Hz were comparable to those of cisplatin, the conversion from monovalent to bivalent adducts appears to be stoichiometric (i.e., all monovalent adducts were converted to bivalent adducts). Thus, as the 300 Hz sensorgram rises to a plateau gain of 50%, the 5 Hz sensorgram drops to a gain of ~0%. We postulate that the signal at 5 Hz remains at ~0% but does not dip further because the majority of oligos on the surface are not platinated to the extent seen with cisplatin, therefore a background population remains for which electron transfer is unaffected.

As additional controls, we evaluated the ability of our platform to respond to two non-platinum antineoplastics: doxorubicin and irinotecan. Doxorubicin intercalates with dsDNA and disrupts topoisomerase-II-mediated DNA repair.^[23] Additionally, doxorubicin can form adducts with DNA under reducing conditions.^[24] When challenging our T₆(AG)₁₀ sensors with this drug, we observed a gain of ~90% in sensorgrams measured at 300 Hz (Figure S6A), an indication that doxorubicin can stack between the tightly packed purine bases at the sensor interface. We also observed a transient response in the 5 Hz sensorgram that may be indicative of adduct formation. This is supported by the fact that methylene blue is a reducing agent that may promote covalent bonding of doxorubicin between two purines^[24] at the interface. Performing a buffer wash after 20 min of T₆(AG)₁₀ exposure to doxorubicin

caused no significant changes in sensor signaling (Figure S6B), supporting the hypothesis that covalently bonded adducts formed which prevented destacking of doxorubicin upon washing. Finally, testing the sensors with irinotecan, a topoisomerase I inhibitor that does not stack with DNA, generated no observable response in the 300 Hz sensorgram (Figure S6C).

To confirm covalent DNA binding to the drugs we studied with our NBEs, we performed liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) measurements on the following samples: one negative solution of 2 μM $\text{T}_6(\text{AG})_{10}$ oligos (Figure S7) and solutions containing the 2 μM $\text{T}_6(\text{AG})_{10}$ oligos plus 100 μM preaquated cisplatin (Figure S8), carboplatin (Figure S9), oxaliplatin (Figure S10), phenanthriplatin (Figure S11), doxorubicin (Figure S12), and irinotecan (Figures S13). Relative to the negative control, cisplatin-, carboplatin- and oxaliplatin-containing solutions all showed masses corresponding to Pt(II) bivalent adducts. The phenanthriplatin-containing solution showed masses corresponding to multiple Pt(II) monovalent adducts. Similarly, the doxorubicin-containing sample showed peaks matched to doxorubicin adducts. Finally, the sample containing irinotecan was the only one not showing evidence of covalent adducts. These observations, although qualitative, are consistent with the results of our NBEs measurements.

The $\text{T}_6(\text{AG})_{10}$ NBE sensor signals can be calibrated relative to drug concentration to determine $\text{EC}_{50\text{s}}$ for platinum(II) drug detection (Figure 4). We illustrate this here for both cisplatin (Figure 4A, C) and phenanthriplatin (Figure 4B, D), but note that similar dose response curves can be built for carboplatin, oxaliplatin, and other platinum(II)-based compounds. Because sensor gain is a function of time after exposure to platinum compounds (Figure 4A, B), we built dose-response curves sampling signals after short ($t \sim 20$ min) and long ($t \sim 2$ h) periods post sensor exposure to preaquated cisplatin and phenanthriplatin (Figure 4C, D). For both sensors, the best sensitivity was obtained after 2 h-long incubations. The EC_{50} for cisplatin – calculated via non-linear regression to the Hill-Langmuir isotherm^[25] – was $13.1 \pm 0.6 \mu\text{M}$. In contrast, a quantitative regression analysis could not be performed for phenanthriplatin because of the biphasic binding kinetics observed with this drug (Figure 4B). Instead, performing the regression analysis between the 100 nM and 2 μM concentration range, we qualitatively estimated an EC_{50} of $\sim 1.3 \mu\text{M}$. Both EC_{50} values are within the same order of magnitude as plasma drug concentrations measured in cancer patients (e.g., $\sim 6 \mu\text{M}$ for cisplatin^[26]), potentially offering a path to develop the $\text{T}_6(\text{AG})_{10}$ NBEs as point-of-care monitors in future work.

The results presented so far for cisplatin and phenanthriplatin were measured after exposure of the $\text{T}_6(\text{AG})_{10}$ NBEs to preaquated complexes, and therefore only reflect DNA binding mechanisms. However, the NBEs can also be used to study full compound activation kinetics, going from the non-aquated cisplatin *cis*-[Pt(NH₃)₂Cl₂] and phenanthriplatin *cis*-[Pt(NH₃)₂-(phen)Cl]⁺, to aquated *cis*-[Pt(NH₃)₂(OH₂)₂]²⁺ and *cis*-[Pt(NH₃)₂-(phen)(OH₂)]²⁺, to their corresponding DNA adducts. We illustrate this ability in chloride-free, HEPES buffer at pH = 7.4 (Figure 5). Under these conditions, the complexes undergo ligand exchange to release chlorides and gain hydroxyl ligands. However, using a neutral pH buffer causes protonation of the hydroxyl ligands (OH) to the activated (OH₂) to be slow.

Serially interrogating $T_6(AG)_{10}$ NBEs every 30 min after addition of *cis*-[Pt(NH₃)₂Cl₂] at 100 μ M resulted in sensorgrams (measured at 300 Hz and 5 Hz) that increased in signal at similar rates over the first 15 h. After this period, the data collected at 300 Hz continued to show a linear increase in sensor signal (purple trace in Figure 5A), while the data measured at 5 Hz decreased (red trace). These observations can be explained as follows: at pH = 7.4, the rate limiting step for cisplatin-DNA adduct formation corresponds to protonation of the hydroxyl ligands following ligand exchange of chloride for hydroxyl, which is necessary for DNA binding. This protonation step slows down the formation of monovalent DNA adducts to a rate that matches the rate of conversion to bivalent adducts. We therefore observe similar signal increases at both frequencies (i.e., mono and bivalent adducts form at similar rates). However, as time progresses, the equilibrium is driven by the conversion of monovalent to bivalent adducts, driving the linear increase in signal seen at 300 Hz, and the concomitant decrease in signal at 5 Hz.

The 5 Hz sensorgram dips below zero because bivalent adduct formation continues to drive the entire population of oligos on the electrode surface to a faster electron transferring conformation. This is in contrast to the measurements performed with *cis*-[Pt(NH₂)₂(OH)₂] at pH = 5.0 (Figure 1), in which the large excess of protons allow rapid protonation of the hydroxyl substituents, driving highly dense platination in the form of both monovalent and bivalent adducts.

Monitoring the interaction of *cis*-[Pt(NH₃)₂-(phen)Cl]⁺ with the $T_6(AG)_{10}$ NBEs we observed a rapid sensor response driven by drug stacking (purple trace in Figure 5B), which occurs at a much faster rate than monovalent adduct formation (red trace). The stacking of *cis*-[Pt(NH₃)₂-(phen)Cl]⁺ via its phenanthridine ligand drives the sensor oligos to fold, causing an increase in sensor signal. This binding interaction dominates so much that at 6 h post drug exposure, the majority of oligos on the sensor surface seem to be in a folded state, causing a plateau response on the 300 Hz sensorgram. However, as some of the stacked molecules slowly dissociate from the sensor oligos, we observed a slow unfolding of oligos that causes a drop of signal at 300 Hz.

At 5 Hz, in contrast, we observed a negative signal plateau over the first 4 h, followed by rapid signal decay. We reason this is because monovalent adduct formation is competing with ligand stacking; the plateau indicates that in the first hours monovalent adducts form relatively fast, but their effect on sensor signal is ultimately overwhelmed by the fast rate of oligo folding via ligand stacking. However, we did not observe signal recovery at 5 Hz, matching the decay observed in the 300 Hz sensorgram (i.e., the signal stayed flat). It is possible that stacked phenanthriplatin molecules that do form monovalent adducts remain stacked, further trapping oligos in a folded state. However, it is also possible that the interrogation frequency of 300 Hz is more sensitive to the fast electron transfer of folded oligos than the interrogation frequency of 5 Hz is of the formation of monovalent adducts. Additional investigations beyond the scope of this work are required to further investigate these specific binding mechanisms, potentially via electrospray droplet impact mass spectrometry.

To evaluate the applicability of using $T_6(AG)_{10}$ NBEs for point of care measurements of cisplatin and phenanthriplatin serum levels, we challenged two independent batches of sensors with each of the drugs (at 100 μ M) in 50% human serum (Figure 6). We used this dilution because, in 100% serum, the high chloride concentration prevented formation of preaquated complexes that are needed to form DNA adducts. Figure S15A, B shows control sensorgrams for measurements performed in 100% serum, which reveal no sensor responses to either drug. When increasing the interrogation time with cisplatin to over 60 h in 100% serum (Figure S15C), we did not observe any signal gain but more signal decay at 5 Hz. In contrast, by diluting the serum by 50% with HEPES buffer (50 mM, containing 100 mM $NaClO_4$) adjusted to pH = 2.1, we obtained samples with half the chloride concentration and a final pH = 5.0. These conditions were conducive to platinum(II)-DNA adduct formation, albeit with slower monovalent adduct formation than sensors performed in HEPES buffer alone because of the still high chloride concentration. Challenging the NBEs with preaquated cisplatin under such conditions, we observed sensorgram responses that indicated the formation of monovalent adducts over the 2 h-long measurement period (red trace in Figure 6A). Bivalent adducts also formed at a lower rate (purple trace). Negative control measurements performed in 50% serum without cisplatin showed stable baselines over the measurement period (Figure 6B). Finally, challenging the sensors with preaquated phenanthriplatin showed a strong monovalent adduct formation rate (red trace in Figure 6C) with much less stacking (purple trace) relative to the experiments performed in pure HEPES buffer at pH = 5.0 (Figure 2). These results illustrate how variations in solution composition and pH can affect platinum(II)-DNA adduct formation mechanisms.

Conclusion

The successful development of nonclassical, next generation platinum complexes with improved antineoplastic activity and lower toxicity strongly depends on our understanding of their reactivity with DNA. Although extensive and quantitative equilibrium and structural studies have revealed key characteristics of platinum(II)-DNA adducts, the transition states that kinetically precede the formation of the terminal DNA adducts are of critical importance to better design such therapeutics. Unfortunately, benchmark methods like ICP-MS cannot resolve fast kinetic processes, can be costly if deferred to a contract research organization (CRO), and require cumbersome sample processing steps that simply do not allow for fast, real-time measurements during high-throughput drug development. As an alternative, nucleic acid-based electrochemical biosensors (NBEs) can be interrogated in real time and, via careful structural design as shown in this work, can be rationally built to selectively study DNA platination mechanisms and kinetics. These sensors are fabricated at a cost of \$10 per measurement or less, and do not require compound extractions or other complex sample preparation steps beyond dilution. Therefore, they offer a convenient and time-resolved approach to study platinum DNA interaction kinetics under various experimental conditions.

Although previous reports by Wu and Lai demonstrated Pt-DNA adducts can be detected in buffered solutions, the short oligo sequence $(AG)_3$ they reported had limited strand flexibility for sensor signaling and limited binding sites. This work, in contrast, demonstrates that NBEs functionalized with the DNA sequence $T_6(AG)_{10}$ achieve superior

signaling output and can distinguish between monovalent and bivalent DNA adduct formation of platinum (II) compounds (e.g., cisplatin, carboplatin, and oxaliplatin) via dual frequency square wave voltammetry. The sensors can also unveil base stacking of non-classical platinum (II) compounds (e.g. phenanthriplatin). Results observed with T₆(AG)₁₀ NBEs match more detailed structural results reported via other more complex, highly specialized techniques (see Table S1). Serial interrogation of the sensors allows for real-time kinetic measurements, and reveals transitions in platinum(II) binding to DNA that inform on the steps preceding platinum(II)-DNA adduct formation. The NBEs are sensitive to platination density and non-covalent drug-DNA interactions, providing great response versatility to elucidate platinum(II)-DNA binding mechanisms and their kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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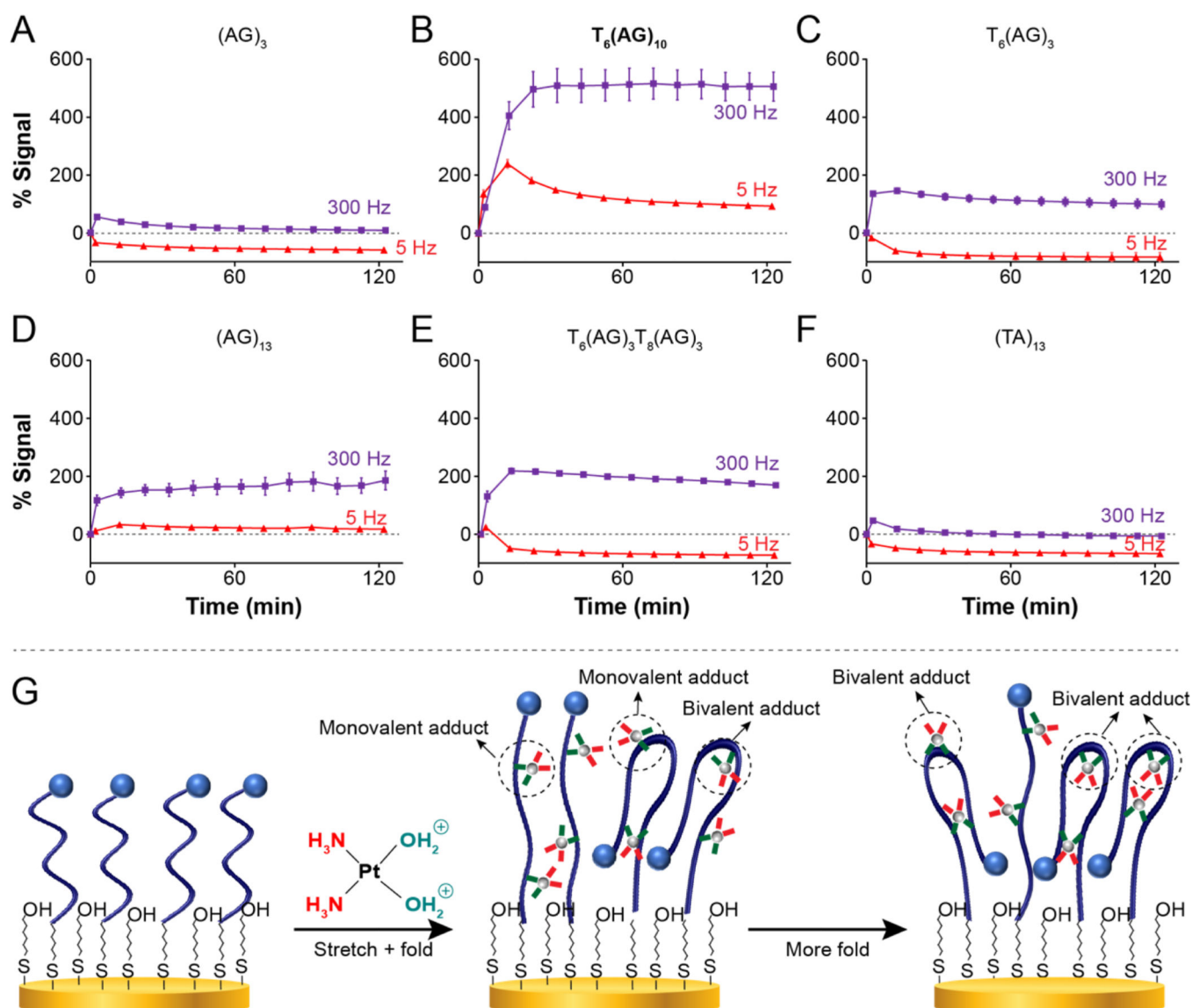


Figure 1.

Cisplatin-DNA adduct formation kinetics monitored via NBEs. (A) NBEs functionalized with 5' hexanethiol- and 3' methylene blue-modified (AG)₃ sequences showed gains, $G_{300\text{Hz}} \sim 8\%$ and $G_{5\text{Hz}} \sim -50\%$ at $t \sim 120$ min. (B) In contrast, NBEs functionalized with the T₆(AG)₁₀ sequence achieved gains, $G_{300\text{Hz}} \sim 500\%$ and $G_{5\text{Hz}} \sim 100\%$. Control NBEs using sequences with (C) less platination sites, T₆(AG)₃; (D) no poly dT linker, (AG)₁₃; and (E) nonconsecutive platination sites, T₆(AG)₃T₈(AG)₃, all achieved lower gains relative to NBEs functionalized with T₆(AG)₁₀. (F) A negative control sequence devoid of platination sites, (TA)₁₃, showed a much lower response to the cisplatin challenge. Solid symbols represent the average measurement from $N = 4$ NBEs, errors represent the standard deviation, and lines are point connectors to highlight data trends. In panels where error bars are not visible, the bars are hidden behind the datapoints (small errors relative to the y axis scale). All data panels show the effect of 100 μM cis-[Pt(NH₂)₂(OH)₂]. Measurements performed in

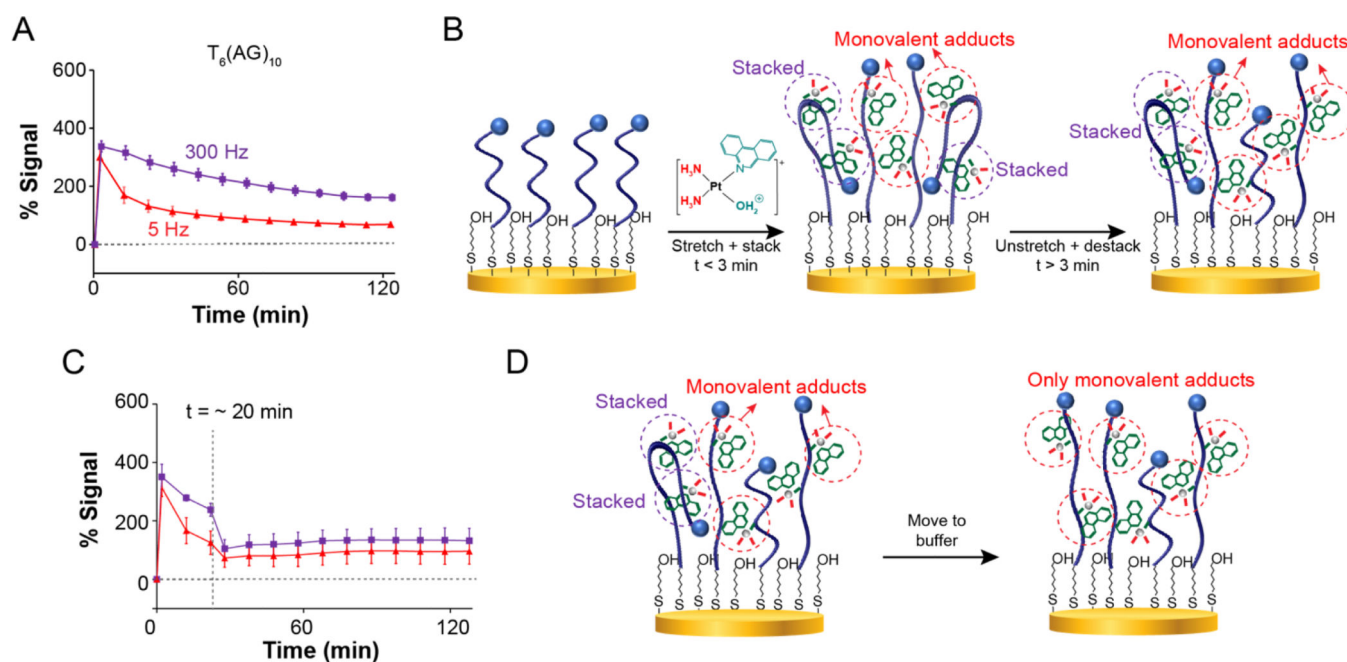
HEPES buffer at pH = 5.0. (G) Postulated mechanism of $T_6(AG)_{10}$ sensor response to $cis-[Pt(NH_2)_2(OH_2)_2]^{2+}$.

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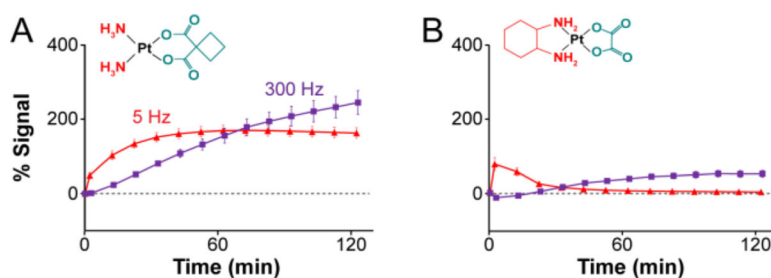
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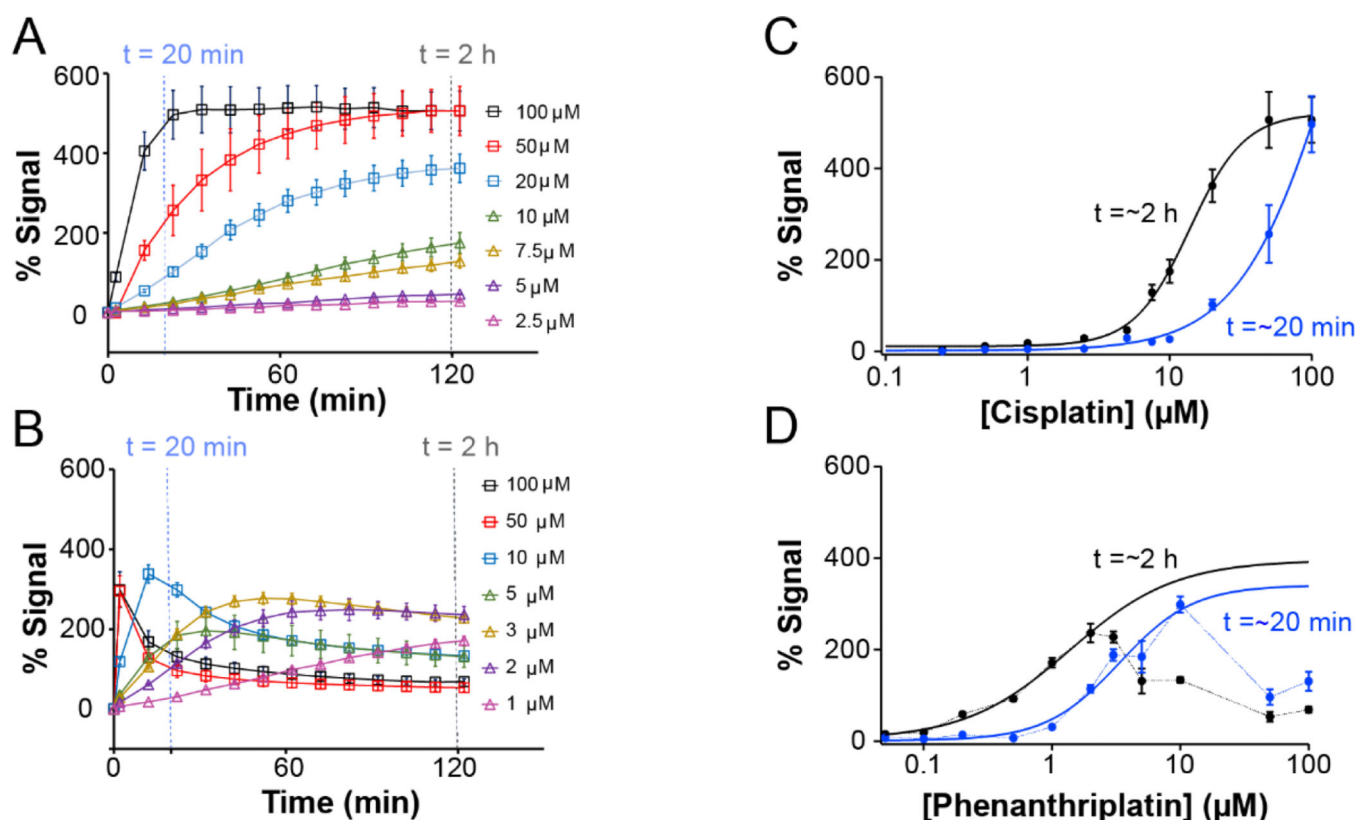
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**Figure 2.**

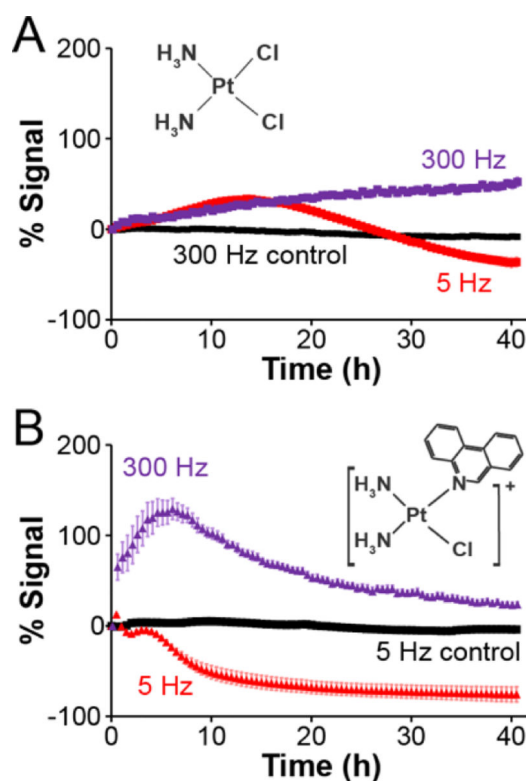
Phenanthriplatin-DNA adduct formation kinetics monitored via NBEs. **(A)** NBEs functionalized with 5' hexanethiol- and 3' methylene blue-modified $T_6(AG)_{10}$ sequences achieved gains of $G_{300\text{Hz}} \sim 160\%$ and $G_{5\text{Hz}} \sim 69\%$ at $t \sim 120$ min. Solid symbols represent the average measurement from $N = 4$ NBEs, errors represent the standard deviation, and lines are point connectors to highlight data trends. Where error bars are not visible, the bars are hidden behind the datapoints (small errors relative to the y axis scale). The data panel shows the effect of *cis*-[Pt(NH₃)₂-(phenanthridine)(OH)]⁺ added to a final concentration of 100 μM . Measurements performed in HEPES buffer at pH = 5.0. **(B)** Postulated kinetic mechanism of $T_6(AG)_{10}$ sensor response to *cis*-[Pt(NH₃)₂-(phenanthridine)(OH₂)]²⁺. **(C)** NBEs that were exposed to preaquated phenanthriplatin for 20 min and then moved into phenanthriplatin-free buffer showed a drop of 200% sensor signal at 300 Hz. These results suggest that 300 Hz is sensitive to stacking, and that stacking interactions can be reversible. However, monovalent adducts remain even after buffer washing, as seen at 5 Hz. **(D)** Postulated reversible phenanthriplatin destacking from $T_6(AG)_{10}$ sensor after switching to phenanthriplatin-free buffer, with retention of monovalent adducts.

**Figure 3.**

Carboplatin- and oxaliplatin-DNA adduct formation kinetics monitored via NBEs. **(A)** T₆(AG)₁₀ NBEs responded to carboplatin with slower kinetics relative to cisplatin (Figure 1B). The sensor gains were: $G_{300\text{Hz}} \sim 244\%$ and $G_{5\text{Hz}} \sim 163\%$ at $t \sim 120$ min. **(B)** When exposed to oxaliplatin, the NBEs sensorgrams presented a stoichiometric conversion of signal from the monovalent adduct seen at 5 Hz to bivalent adduct seen at 300 Hz, with net gains at $t \sim 120$ min of $G_{300\text{Hz}} \sim 50\%$ and $G_{5\text{Hz}} \sim 3\%$. The $G_{300\text{Hz}}$ gain was significantly lower than that observed with cisplatin. Solid symbols represent the average measurement from $N = 4$ NBEs, errors represent the standard deviation, and lines are point connections to highlight data trends. In panels where error bars are not visible, the bars are hidden behind the datapoints (small errors relative to the y axis scale). Data panels show the effect of carboplatin or oxaliplatin, respectively, spiked to a final concentration of 100 μM . Measurements performed in HEPES buffer at pH = 5.0.

**Figure 4.**

Cisplatin and phenanthriplatin dose-response curves for $T_6(AG)_{10}$ NBEs. Sampled sensor signal across drug concentrations ranging from 100 nM to 100 μ M, with pre-equated (A) cisplatin and (B) phenanthriplatin (see full range of measurements in Figure S14A, B). Symbols represent the average measurement from $N = 4$ NBEs, errors represent the standard deviation, and lines are point connections to highlight data trends. (C) For cisplatin, the lowest EC_{50} was observed when sampling signal at $t \sim 2$ h after drug exposure ($EC_{50} = 13.1 \pm 0.6$ μ M). Data measured at 300 Hz to only show bivalent adduct formation. (D) For phenanthriplatin, the sensors did not reach a plateau, but sensor sensitivity was also lower at $t \sim 2$ h (estimated $EC_{50} \sim 1.3$ μ M). Data shown at 5 Hz to only show monovalent adduct formation. In panels C and D, solid symbols represent the average measurement from $N = 4$ NBEs, errors represent the standard deviation, and solid lines show non-linear regression to a Hill-Langmuir isotherm. Dashed lines in Panel D are point connectors to facilitate data interpretation. In panels where error bars are not visible, the bars are hidden behind the datapoints (small errors relative to the y axis scale). Measurements performed in HEPES buffer at pH = 5.0.

**Figure 5.**

Simultaneous study of aquation and DNA adduct formation kinetics. These sensorgrams were recorded in chloride-free HEPES buffer at pH = 7.4. **(A)** T₆(AG)₁₀ NBEs responded to the addition of *cis*-[Pt(NH₃)₂Cl₂] over a 40 h-long period. Under these conditions, monovalent and bivalent adducts formed at similar rates over the first 15 h of continuous monitoring. After 15 h, however, the bivalent adducts dominated the overall signal output as seen by the linear increase in gain at 300 Hz and concomitant decrease of signal at 5 Hz. **(B)** With *cis*-[Pt(NH₃)₂(phen)Cl]⁺, the NBEs showed a rapid response at 300 Hz caused by stacking of the phenanthridine ligand in the T₆(AG)₁₀ oligos. This response reversed to a signal close to baseline after 40 h of continuous monitoring. Some DNA-phenanthridine complexes slowly formed monovalent adducts, resulting in the plateau seen at 5 Hz over the first 4 h. After this period, however, phenanthriplatin stacking seemed to strongly drive the folding of oligos, as seen by the signal decrease over time. Solid symbols represent the average measurement from *N* = 4 NBEs, errors represent the standard deviation, and black symbols represent control measurements in the absence of platinum(II) compounds.

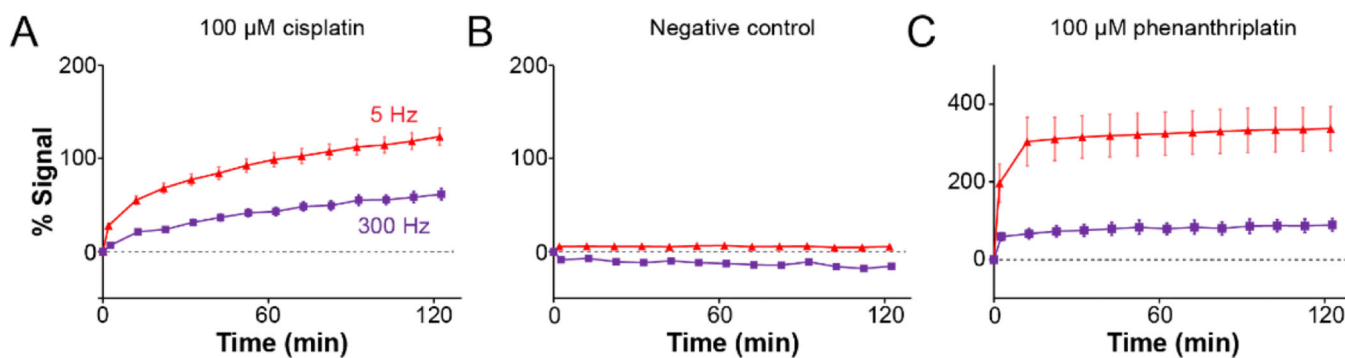


Figure 6.

Cisplatin- and phenanthriplatin-DNA adduct formation kinetics in 50% serum. These sensorgrams were recorded in human serum diluted by 50% with HEPES buffer, with a final pH = 5.0. **(A)** $T_6(AG)_{10}$ NBEs responded to the addition of preaquated cisplatin. Monovalent adduct formation dominated over the recording period as shown by the larger magnitude of the 5 Hz sensorgram relative to the 300 Hz sensorgram. **(B)** Negative control without addition of preaquated cisplatin, to show stable sensor baselines at both frequencies. **(C)** Sensor responses to preaquated phenanthriplatin. Monovalent adduct formation is dominant under these conditions, with $G_{5\text{Hz}} \sim 380\%$. Stacking only produced gains of $G_{300\text{Hz}} \sim 80\%$. Solid symbols are the mean from $N = 4$ NBEs, errors represent the standard deviation, dashed lines indicate 0% signal.

Table 1.

Oligonucleotide sequences and modifications used in this work.

Abbreviation	Sequence
(AG) ₃ ^[a]	5'-HS-C ₆ -PO ₄ -AGA GAG-PO ₄ -MB-3'
T ₆ (AG) ₃	5'-HS-C ₆ -PO ₄ -TTT TTT AG AG AG-PO ₄ -MB-3'
T ₆ (AG) ₁₀	5'-HS-C ₆ -PO ₄ -TTT TTT AGA GAG AGA GAG AGA GAG AG-PO ₄ -MB-3'
(AG) ₁₃	5'-HS-C ₆ -PO ₄ -AGA GAG AGA GAG AGA GAG AGA GAG AG-PO ₄ -MB-3'
T ₆ (AG) ₃ T ₈ (AG) ₃	5'-HS-C ₆ -PO ₄ -TTT TTT AGA GAG TTT TTT TTA GAG AG-PO ₄ -MB-3'
(TA) ₁₃	5'-HS-C ₆ -PO ₄ -TAT ATA TAT ATA TAT ATA TAT ATA TA-PO ₄ -MB-3'

^[a]Original sequence reported by Wu and Lai.^[19]