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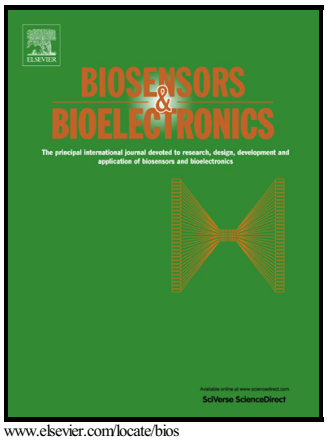

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Electrochemical current rectification – a novel signal amplification strategy for highly sensitive and selective aptamer-based biosensor

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ABSTRACT

Electrochemical aptamer-based (E-AB) sensors represent an emerging class of recently developed sensors. However, numerous of these sensors are limited by a low surface density of electrode-bound redox-oligonucleotides which are used as probe. Here we propose to use the concept of electrochemical current rectification (ECR) for the enhancement of the redox signal of E-AB sensors. Commonly, the probe-DNA performs a change in conformation during target binding and enables a nonrecurring charge transfer between redox-tag and electrode. In our system, the redox-tag of the probe-DNA is continuously replenished by solution-phase redox molecules. A unidirectional electron transfer from electrode via surface-linked redox-tag to the solution-phase redox molecules arises that efficiently amplifies the current response. Using this robust and straight-forward strategy, the developed sensor showed a

substantial signal amplification and consequently improved sensitivity with a calculated detection limit of 114 nM for ATP, which was improved by one order of magnitude compared with the amplification-free detection and superior to other previous detection results using enzymes or nanomaterials-based signal amplification. To the best of our knowledge, this is the first demonstration of an aptamer-based electrochemical biosensor involving electrochemical rectification, which can be presumably transferred to other biomedical sensor systems.

Keywords: electrochemical current rectification, aptamer, signal amplification, electrochemical biosensor

1. Introduction

In recent years, one emerging research interest in biosensor is using aptamers as molecular recognition element, which are usually single-strand DNA/RNA molecules that can recognize their corresponding targets with high affinity and selectivity (Lee et al., 2008; Tan et al., 2013). Compared to antibodies, they are usually selected through in vitro SELEX procedures, as well as chemically synthesized with diverse functional modifications. Aptamers are generally thermostable, fold reversibly, and are inexpensive (Palchetti et al., 2012). Various aptamer-based biosensors have been developed based on different detection principles including optical, electronical, and electrochemical detection *etc*. Among them, electrochemical devices have their own advantages, such as being easily miniaturizable and highly sensitive, possessing simple operation modes, and supporting point of care diagnostics (Palchetti et al.,

2012; Willner et al., 2007). Electrochemical DNA/aptamer-based (E-DNA/E-AB) sensors represent a recently developed class of sensors where the binding of target alters the conformation of an electrode-bound, redox-tagged oligonucleotide, which leads to a detectable Faradaic current response (Baker et al., 2006; Lubin et al., 2010; Radi et al., 2006; Xiao et al., 2007; Xiao et al., 2007). These folding-based sensors have been used to detect a wide range of targets. They usually possess a rapid response (seconds to minutes), and are able to detect subpicomolar to micromolar concentrations (Lubin et al., 2010). However, the obtained electrochemical signals are usually limited by the surface probe density, which is typically in the range of a few pmol/cm^2 or even less (White et al., 2008). Therefore, simple and versatile strategies are required that facilitate a distinct amplification of the sensor signal. Different signal amplification methods have been proposed that predominantly rely on the use of enzymatic reactions for instance horseradish peroxidase (Liu et al., 2008), polymerase-mediated rolling circle amplification (Cho et al., 2005), exonuclease catalyzed target recycling, *etc.*(Hsieh et al., 2010) or the use of different kinds of multi-step nanomaterial assisted amplification through enhancing numerous binding units or electroactive probes (Chen et al., 2014; Huang et al., 2014; Palchetti et al., 2012). All these approaches require comparably expensive enzymes, stringent environmental conditions or complicated synthesis processes (Chen et al., 2014; Cho et al., 2005; Hsieh et al., 2010; Huang et al., 2014; Lee et al., 2008; Liu et al., 2008). Therefore, there is still a strong demand for the development of simple signal amplification methods for aptamer-based electrochemical sensors.

Here, we report on the use of electrochemical current rectification (ECR) concept for the enhancement of redox signal of an electrochemical aptamer-based sensor. Electrochemical current rectification has been first reported by Murray *et al.*(Abruna et al., 1981; Denisevich et al., 1981). A unidirectional current can be realized by facilitating the transfer of electrons between solution phase redox probes and the electrode mediated by surface tethered charge transfer groups, while the transfer in the reverse direction is prohibited for thermodynamic reasons. Usually the direct charge transfer between redox probe in solution and electrode is blocked by a supporting insulating layer embedding the charge transfer mediators. Consequently, the entire charge transfer has to occur via these surface confined redox groups which control the electrochemical behavior of whole system (Chidsey et al., 1986). Various ECR-based device concepts have been reported using synthetic redox active molecules, polymers, as well as ferrocene-labeled biomolecules such as streptavidin which could efficiently facilitate unidirectional current flow (Azzaroni et al., 2008; Rong et al., 1990). We have demonstrated that the redox properties of biomolecules can be used directly to realize ECR effects by utilizing the redox active microperoxidase (MP-11) as charge transfer mediator (Liu et al., 2010a). Furthermore, electrochemical rectifications have been used to perform molecular logic gate mimicking operations and to realize transistor functions by implementing an independent gate electrode (Liu et al., 2010b; Liu et al., 2013). Recently, an electrochemical biosensor using the ECR scheme was demonstrated that facilitates the detection of cancer cells with sensitivity as low as 10 cells mL^{-1}(Li et al., 2012). With the motivation to implement the emerging scheme of

electrochemical rectification into aptamer-based biosensor concept, we have investigated the signal amplification capabilities of redox tagged aptamer sensors, which to the best of our knowledge remains challenging so far.

As shown in Scheme 1, we adopt the ECR-based signal amplification process to an ATP targeting aptamer as model system. ATP is vital to living organisms and provides the support for the metabolism in biological tissues. Thus the detection of ATP is substantial important in various fields of live sciences, for instance, the energy metabolism, intra- and extracellular signaling, clinic diagnosis (Larsen et al., 1974; Perez-ruiz et al., 2003). Upon binding of the target ATP, the induced conformational change of surface tethered aptamer will alter the electron tunneling between the aptamer associated ferrocene (Fc) groups and the sensing electrode. Without signal amplification, the resulting current response is usually relatively small and limited by the amount of probe molecules on surface, typically about pmol/cm^2. A solution-phase redox probe, e.g. K_2IrCl_6, can be used to continuously replenish the charge transfer mediator with charge carriers (Scheme 1). According to the relative redox potentials of Fc (0.32 V) and iridate (IV/III) ions (0.71 V vs. Ag/AgCl), a unidirectional electron transfer from the electrode via the ferrocene group to the solution phase redox group arises that efficiently amplifies the cathodic current response. In the reverse direction, the electron transfer process is thermodynamically forbidden since the electrons cannot be transferred from hexachloroiridate to Fc (Li et al., 2012; Liu et al., 2010a; Liu et al., 2010b). Using this robust and straightforward strategy, we are able to detect the presence of our target with a substantial signal amplification and consequently

improved sensitivity. We determined a calculated detection limit of 114 nM, which was improved about 10 times compared with the amplification-free strategy. The result was also better than other previous reported ATP aptamer-based electrochemical sensors (Zayats et al., 2006; Zuo et al., 2007; Zuo et al., 2009), as well as superior to the results using enzymes or nanomaterials-aimed signal amplification (Cho et al., 2005; Kashefi-Kheyrabadi et al., 2012; Hsieh et al., 2006; Liu et al., 2012).

2. Experimental Section

2.1.Reagents

ATP aptamer sequences were all synthesized by FRIZ Biochem (Neuried, Germany) with the sequences of 5′-Ferrocene-$(CH_2)_6$-ACC TGG GGG AGT ATT GCG GAG GAA GGT-$(CH_2)_6$-SH-3′. The oligonucleotide concentration was determined by UV-vis spectroscopy using the absorption at 260nm. 1-undencanethiol (UDT), tris-(2-carbozyethyl) phosphine hydrochloride (TCEP), dodecyltrimethylammonium bromide (DTAB), potassium hexachloroiridate (IV) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. All aqueous solutions were prepared using ultra-pure water (18.2 MΩ, Milli-Q, Millipore).

2.2 Electrode cleaning and preparation

The aptamer modified sensor was fabricated by using Au (111) crystal electrodes, which was firstly annealed with a hydrogen flame and then cooled to room temperature. The surface area was determined in 5 mM H_2SO_4 electrolyte before modification with the thiolated Fc-tagged aptamer sequence. DNA oligomer was pretreated with 10 mM TCEP for 1 h at room temperature to reduce the disulfide bond

of the probe DNA. The clean gold surface was incubated with mixture solution composed with 0.5 μM thiolated Fc-labeled DNA oligomer, 1 mM UDT, 50 mM DTAB in high salt phosphate buffered saline (100 mM PBS, 1.5 M NaCl, 1 mM $MgCl_2$, pH=7.4), overnight, at room temperature. The mixted self assembled monolayer could form through thiol-gold bond between electrode surface and thiolated molecules. In our investigation, DTAB was added to solubilize UDT in PBS solution. The surface was then rinsed with buffer (10 mM PBS, 200 mM NaCl, 15 mM KCl, pH=7.4) and stored in the 10 mM PBS buffer prior to measurement.

2.3.Electrochemical measurements

Cyclic voltammetry (CV) and alternating current voltammetry (ACV) were performed by means of an Autolab potentiostat/galvanostat instrument with conventional three-electrode configuration comprised of a platinum wire as the auxiliary electrode, Ag/AgCl as the reference electrode, and the modified gold used as working electrode. For the CVs the electrode potential was scanned in PBS (10 mM PBS, 200 mM NaCl, 15 mM KCl, pH 7.4) from 0.8 to -0.2 V and then returned to 0.8 V, with a scan rate of 50 mV/s. ACV measurements were conducted with an amplitute of 5 mV, and a frequency of 10 Hz.

The surface area of electrode was determined by CV curve in 50 mM H_2SO_4 solution from the area of the gold oxide reduction peak, while the surface coverage was calculated using a previously established relationship with ACV peak current described before (Feng et al., 2012): I_{avg} (E_0) = $2n\mathit{f}FN_{tot}[\sinh(nFE_{ac}/RT)]/[\cosh(nFE_{ac}/RT)+1]$. In which I_{avg} (E_0) is the average peak

current in the AC voltammograms, n is the number of electron transferred per redox event (with Fc lable n=1), *f* is the frequency of the applied ACV perturbation, F is the Faraday constant, N_{tot} is the number of electroactive DNA probe in moles, E_{ac} is the peak amplitude (E_{ac} = 25 mV in experiment), R is the universal gas constant, and T is the temperature (T=288.15 K) (O'Connor et al., 1999; Sumner et al., 2000).

For all detection measurements in buffer, targets were diluted with 10 mM PBS buffer into different concentration, except for the time-course study. And then the modified electrodes were incubated for 20 min in one solution among above different concentrations at room temperature. Prior to measurement, rinsing with the measured buffer is necessary. All data points and error bars represent the average and standard deviations from three independently electrodes.

For the recovery measurements in serum, different ATP concentrations were spiked into 100-diluted blank fetal bovine serum (FBS), and the other processes were same as in buffer detection. Also, 1 mM ATP were added into 100% and 50% FBS for further evaluate the selectivity of our sensor.

3. Results and discussion

A 27-mer ATP aptamer comprising a redox-active ferrocene at its 5′-terminus was covalently attached to a gold electrode via a thiol-gold bond at its 3′-terminus. The characterization of the sensor was performed as we described previously (Feng et al., 2012a; Feng et al., 2012b), except that 1-undencanethiol (UDT) was used instead of 6-mercaptohexanol (MCH) as diluent agent to achieve more compact and efficient

blocking layer on the surface (Liu et al., 2010b). In the absence of the target ATP, the immobilized aptamer was probably randomly folded in low salt solution with Fc far away from the electrode which resulted in a small redox current response (Figure 1A, line a) (Scheme 1, left). The addition of 1 mM ATP to the solution would induce a change of the aptamer conformation upon ATP binding and reduce the distance between terminal Fc groups and electrode surface. This proximity enhanced the charge transfer and increased the current of the Fc associated redox peaks at around 0.32 V (vs.Ag/AgCl) (Figure 1A, line b). After adding 1 mM K_2IrCl_6 to the system, the cathodic current significantly increased due to an ECR-based current amplification while the anodic current remained small (Figure 1B). Electrons are efficiently relayed from the electrode via the Fc center to iridate (IV) ions, which leads to a significantly increase of the cathodic current and thus to an enhancement of the sensor signal. Regarding current rectification, the ATP molecules act as concentration dependent chemical gate that modulates the rectified current by enabling a charge transport channel between electrode and solution phase redox probes due to a conformational change of the aptamer. The effective blocking of the direct redox reaction of Ir (IV/III) with the electrode by UDT is exemplified by the missing anodic current response, which contrasts systems where the commonly used MCH agent employed with iridate (IV/III) peaks around 0.71 V (vs. Ag/AgCl). (Figure 1C).

To fully exclude the background signal influence, we compared here the relative Faradic current change ΔI, defined as $(I_{target}\text{-}I_{apt\text{-}AuE})/I_{apt\text{-}AuE}$ ($I_{apt\text{-}AuE}$ means current of aptamer modified gold electrode before target binding), for conventional aptamer and

ECR-aptamer ATP detection at the same concentration (Figure 1). The ΔI value increased from 0.85 (curve a,b) to 6.1 (curve c, d) from CV results, with a 7.1-fold increase after ECR amplification (Figure 1B). We further used another electrochemical method, alternating current voltammetry (ACV) measurement, to compare the current changes with ECR-based signal amplification, which supposed to be sensitive for surface changes of low coverage samples (Figure 1D) (Feng et al., 2012). The ΔI of the aptamer-based sensor was calculated to be 1.32 (curve a, b), while this value increased to 5.2 in the presence of solution-phase redox probe K_2IrCl_6. This 4-fold increase is ascribed to the ECR-based signal amplification effect (curve c, d).

The obtained signal amplifications base on a careful optimization of the experimental conditions. We adjusted the density of surface tethered aptamer molecules within the self-assembled monolayer to ensure on the one hand that we had sufficient redox mediators on the surface for a strong sensor signal and on the other hand that the appropriate space for folding induced by the target. From plotting the current signal versus the surface coverage of the aptamer, we found an optimal aptamer coverage of 35.3 ± 2.58 pmol/cm^2 which was obtained for a 0.5 μM aptamer modification (Figure 2A and Figure S1). Since the number of aptamer molecules on electrode surface is relatively small, the observed current of the sensor following the conventional sensor scheme is directly restricted by the surface coverage effect (Hsieh et al., 2006; White et al., 2008). In our system, solution-phase redox probes were diffusing to the electrode, continuously replenish the charge transfer mediator, and thus amplify the

current response. There is a linear relationship between current and the logarithm of scan rate, indicating a diffusion-controlled process (Figure 2B and its inset). The homogenous mixing of redox active aptamer molecules and blocking 1-undencanethiol (UDT) molecules were proven by desorption experiment performed in KOH solution. We observed only a single peak at around -0.92V for desorption of both molecules with a homogenous aptamers and UDT mixed molecules, indicating that the aptamers were homogenously mixed with UDT molecules (Figure S2) (Liu et al., 2013). An incubation time of 20 min was chosen for the application of the target in order to get a saturation of the signal which was represented by a plateau within time dependent experiments (Figure 2C). A frequency of 10 Hz generated the highest signals for ACV measurements (Figure S3). A saturation of the electrochemical signal at the concentration of 1 mM redox probe K_2IrCl_6 was also found in the presence of ATP target (Figure 2D), therefore the solution-phase probe concentration was chosen as 1 mM for the following measurements.

Under the optimized experimental conditions, the aptamer-based sensor exhibited sensitive response at a broad concentration range of target molecules as shown in Figure 3A. To demonstrate the sensitivity improvement by the ECR-based method, we directly compared the detection limits achieved in the absence and presence of ECR amplification. When the ATP concentration varied between 0 to 150 μM, the ECR-based sensor sensitively responded to the change of the ATP concentration, Figure 3B. A linear relationships were fitted at low ATP concentrations (0 to 5 μM) with a calculated detection limit (3σ/S, in which σ is the standard deviation for the

blank solution, and S is the slope of the calibration curve) of 114 nM (R^2=0.98, n=3) (Figure 3B and inset, line a). In contrast, the detection limit was 1.09 μM when the aptamer-based sensor was adopted without amplification (Figure 3B inset, line b and Figure S4 in the Supporting Information). This leads to a sensitivity enhancement of 9.6 times due to the ECR-based signal amplification, which is superior to the 5-fold improvement with exonuclease catalyzed amplification (Hsieh et al., 2006). The detection limit with ECR signal amplification was lower than the previously reported results for amplification-free ATP aptamer-sensors (Zayats et al., 2006; Zuo et al., 2007; Zuo et al., 2009) as well as fluorescent chip arrays with aptazyme-catalyzed ligation and rolling circle amplification (Cho et al., 2005), of which the detection limits were all around 1 μM. The detection limit is also comparable with other ATP detection limits determined by optical, electrochemical, and transistor sensors etc., as details summarized in Table 1. However, our sensor concept relies on a simple and robust detection scheme using aptamers as recognition motif, which is generally thermostable and inexpensive. Furthermore, this novel ECR-based amplification also owns straightforward operation compared with others enzyme or nanomaterial-based amplification procedure (Cho et al., 2005; Kashefi-Kheyrabadi et al., 2012; Hsieh et al., 2006; Liu et al., 2008; Lu et al., 2010; Song et al., 2009).

Since neither the tethered nor the solution phase redox probes impair the selective binding between aptamer and its target, the designed sensor also own excellent selectivity among others analogs, as well as good performance in complicated serum solution. The target specific binding properties of aptamer facilitates the sensor

response selectively in the presence of ATP among other organic triphosphate analogues, such as cytidine triphosphate (CTP), guanosine triphosphate (GTP), thymine triphosphate (TTP), and uridine triphosphate (UTP). As shown in Figure 4A and Figure 4B, the changes in electrochemical response induced by the non-specific binding are significantly smaller than that caused by the specific binding of ATP, even at 5-fold excess of the ATP analogues, providing high sensitivity and selectivity for target molecules than its controls. This superior property of our ECR-aptamer sensor is conserved even in complicated serum solution (Figure 4C). The recovery experiment with different ATP concentrations was carried out to evaluate the aptasensor response. Four different added ATP concentrations were spiked in 100-diluted fetal bovine serum (FBS) instead of buffer alone, and other experimental conditions were same. As shown in Table 2, the recoveries of ATP in the diluted serum were 95.7~109.2%. This results suggested that the sensor can still work in this complex matrix. We further measured a relative electrochemical signal change ΔI of 4.67 and 3.2 in 50% and 100% FBS with background signal subtracted, respectively, in the presence of 1 mM ATP molecule. In addition, our sensor showed an excellent stability when stored in 10 mM PBS solution. 85.6 % of original signal was retained even after 15 days storage (Figure 4D) at room temperature, which is hardly feasible by means of ELISA.

4. Conclusion

In conclusion, we demonstrate here a novel, inexpensive, and robust strategy to amplify the signal of an electrochemical aptamer sensor by means of a relayed charge transfer between solution phase redox probes and surface tethered redox labeled aptamer molecules. Therefore, we applied the principles of electrochemical rectification to an ATP targeting aptamer model-system. For the resulting ECR-based aptamer sensor, current signal amplification was realized and a detection limit as low as 114 nM was determined, which corresponds to an improvement of about ten times compared to the amplification-free detection, as well as superior to other previous reports. The selectivity and robustness of our sensor were proven by demonstrating excellent performance in complicated serum solution and storing the sensor in medium for several weeks. It is expected that this universal ECR-based signal amplification scheme could be applied to aptamers targeting other molecules and thereby effectively foster the development of novel aptamer-based electrochemical biosensor.

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Figure Captions

Scheme 1. Schematic illustration of the electrochemical current rectification based signal amplification for aptamer-based electrochemical biosensor. DNA aptamer and blocking agent UDT are modified on the surface through Au-S bonds. Relayed charge transport from electrode via aptamer linked ferrocene group (0.32V vs.Ag/AgCl) to solution phase redox probes (0.71V vs.Ag/AgCl).

Figure 1. Electrochemical responses with ECR-based signal amplification. (A) CV curves of aptamer modified electrodes with (a) 0 mM ATP, (b) 1 mM ATP; (B) CV curves of (a) 0 mM ATP, (b) 1 mM ATP, (c) 0 mM ATP with 1 mM K_2IrCl_6, and (d) 1 mM ATP with 1 mM K_2IrCl_6, using UDT as blocking agent. (C) CV curves of aptamer modified electrode modified with MCH by targeting 1 mM ATP (black), and further with the presence of 1 mM K_2IrCl_6 (red). (D) ACV curves of different aptamer modified electrodes, (a) 0 mM ATP, (b) 1 mM ATP, (c) 0 mM ATP with 1 mM K_2IrCl_6, and (d) 1 mM ATP with 1 mM K_2IrCl_6, all above using UDT for modification.

Figure 2. Optimization of different experimental conditions. (A) The surface coverage of aptamer probe for optimal modification. (B) The diffusion-controlled process. CV curves of aptamer-modified electrode in 1 mM $K_2Ir_2Cl_6$ solution at different scan rates, inset: the linear relationship between current intensity and the logarithm of scan rate. Scan rates: 5, 25, 50, 75, 100, 150, 250, 500, 750, 1000 mV/s. (C) Incubation time dependence. The signal response was measured after incubation in 1 mM ATP solution at different times to facilitate the aptamer conformation change. 20 min was finally chosen as optimal time for following experiments. (D) K_2IrCl_6 concentration optimization recorded from solutions of different concentrations: 50, 200, 1000, 2000 and 5000 μM.

Figure 3. (A) Electrochemical current responses of ECR-based sensor with different ATP concentrations from 0 μM to 150 μM. (B) Concentration dependence of the current on ATP concentration with ((a) black dots) and without ((b) red dots) ECR-based signal amplification, and the error bars were obtained through three independent measurements. Inset: magnification of the linear range and linear relationships were fitting with 95% confidence level.

Figure 4. Selectivity of the prepared biosensor for ATP detection. (A) ACV responses of different target molecules, 1 mM ATP (black) and 5 mM other analogues, GTP (red), TTP (blue), UTP (dark cyan), and CTP (magenta). (B) The column comparison image of target responses. (C) Electrochemical current responses of ECR-based sensor in complicated solutions, (a) 50% FBS, no ATP; (b) 50 % FBS with 1 mM ATP and (c) 100% FBS with 1 mM ATP. (D) The stability of modified electrode while keeping for 15 days stored in PBS solution in room temperature.

Table 1. Comparison of the present work with other reported aptamer-based ATP sensors.

Different detection methods		Detection limits	References
Enzyme-aimed amplification	Rolling circle amplification	1 μM	Cho et al. (2007)
	Exo III amplification	0.25 μM	Liu et al. (2012)
Nanomaterials-aimed amplification	Silver nanoparticles conjugated electrochemical sensor	1 μM	Kashefi-Kheyrabadi et al. (2012)
	Magnetic SiO_2 sensor	0.1 μM	Song et al. (2009)
	Graphene-based electrochemical aptsensor	0.015 μM	Wang et al. (2012)
	Graphene two proton sensor	0.5 μM	Yi et al. (2014)
	Core–Shell Ag@SiO_2 Nanoflares sensor	8 μM	Lu et al. (2014)
Other methods	G-quadruplex aptasensor	1μM	Zhang et al. (2012)
	Sandwiched aptamer sensor	1 μM	Zuo et al. (2007)
	Ion-selective field-effect transistor	1 μM	Zayats et al. (2006)
	Electrochemiluminescence sensor	0.64 μM	Liu et al. (2010c)
	Surface plasmon resonance	0.010 μM	Xie et al. (2014)
	Quartz crystal microbalance	6 μM	Ozalp et al. (2011)
Present work		0.114 μM	

Table 2. The recovery experiment of ATP in 100-diluted FBS samples.

Samples	Added (nM)	Found (nM)	Recovery (%)	RSD (%) (*n*=3)
1	100	108.4±28.4	108.4	28.4
2	200	191.3±15.8	95.7	7.9
3	300	322.6±13.8	109.2	4.6
4	500	531.5±48.1	106.3	9.6

Highlights

We propose here a novel concept that uses electrochemical current rectification for signal amplification of electrochemical aptamer-based sensor.

Solution-phase redox probes are adopted to continuously replenish a charge transfer mediator and amplify the current response with a unidirectional electron transfer.

The detection limit for the target is improved by one order of magnitude compared with the amplification-free detection and superior to other previous detection results.

This robust and straightforward strategy pave the way for new electrochemical signal amplification strategies and novel applications for electronic elements in biomedical diagnosis.

Scheme 1:

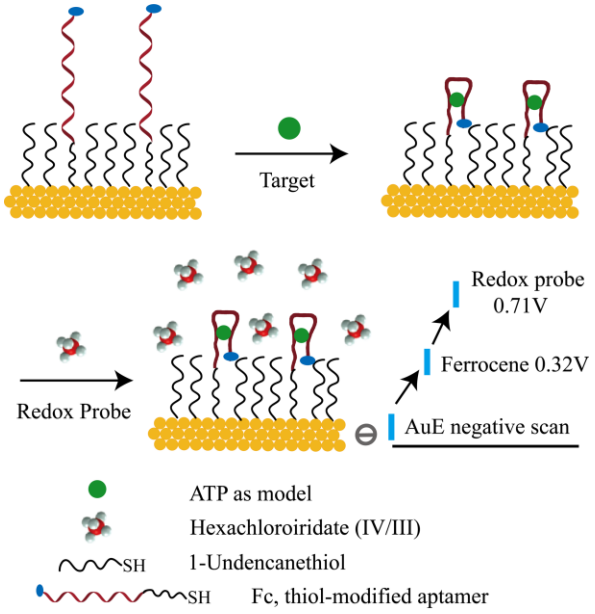

Figure 1

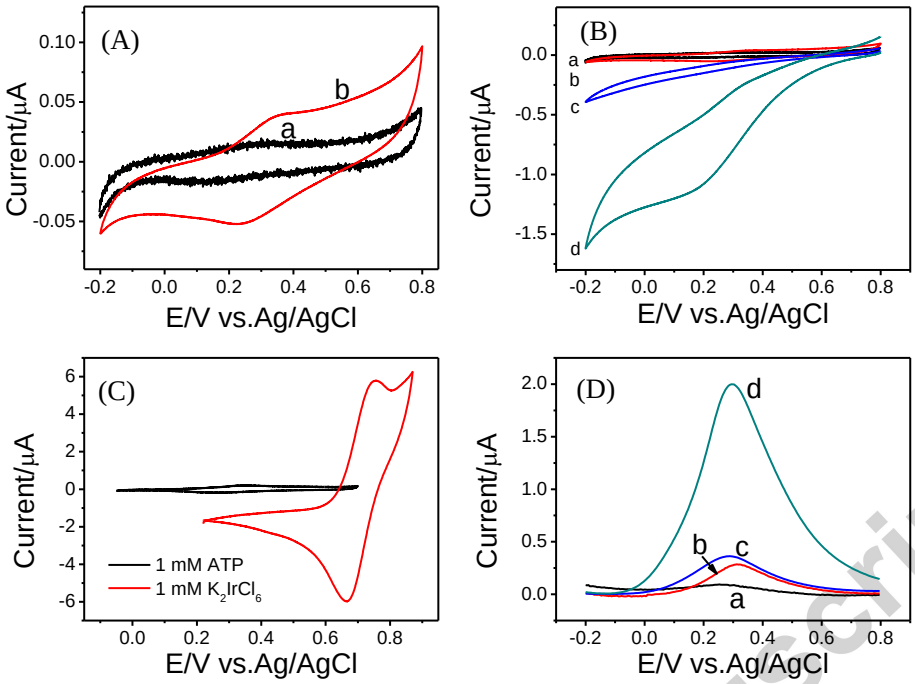

Figure 2

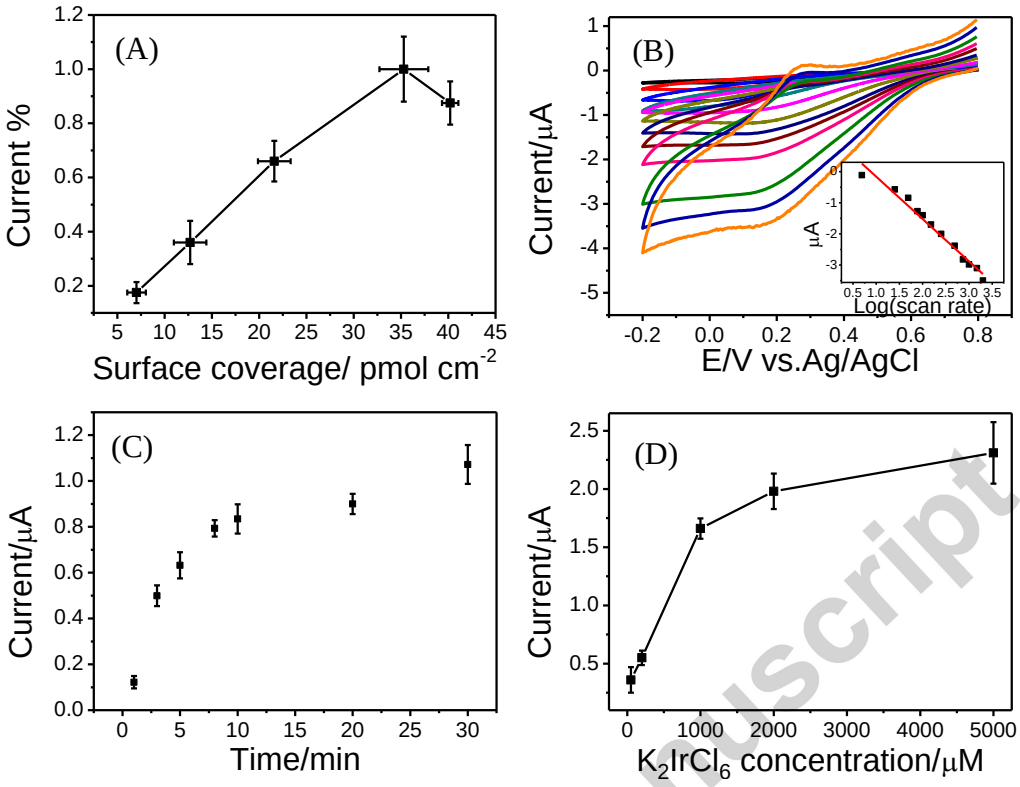

Figure 3

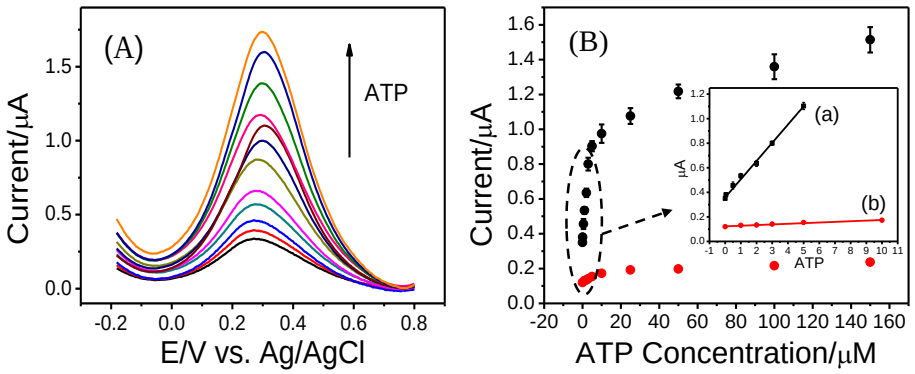

Figure 4