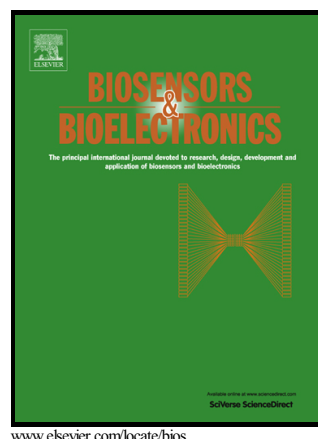


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Title

Ultrasensitive electrochemical detection of engrailed-2 based on homeodomain-specific DNA probe recognition for the diagnosis of prostate cancer

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Abstract

It is well known that the engrailed-2 (EN2) protein, a biomarker for prostate cancer, strongly binds to a specific DNA sequence (5'-TAATTA-3') to regulate transcription. Based on this intrinsic property, DNA probes with additional flanked sequences were designed and optimized. Various measurements, such as an electrophoresis mobility shift assay, a surface plasmon resonance, and a quantitative fluorescence assay were performed to investigate the feasibility of the DNA probes. Then, the affinities of the DNA probes to the target protein were quantitatively determined using FAM-modified DNA probes and magnetic beads, resulting in dissociation constants ranging from 61.03 to 98.84 nM. To develop an early diagnosis platform for prostate cancer, an ultrasensitive electrochemical biosensor based on the electrodeposition of gold nanoparticles was designed. The EN2 protein was quantitatively detected using the electrochemical biosensor, and the calculated detection limit was found to be 5.62 fM. Finally, the specificity and applicability of the biosensor were verified using several proteins and an artificial urine medium. The impedance signals increased in the cases of EN2, suggesting that the system exhibited high selectivity to only EN2.

Keywords

Engrailed-2; Prostate cancer; Early diagnosis; Homeobox; Impedance spectroscopy; Fluorescence

1. Introduction

Prostate cancer is the most frequently diagnosed cancer and the second major cause of cancer-related death in men (Siegel et al. 2011). Early screening and diagnosis of prostate cancer are required to reduce its mortality rate. There are several diagnostic methods for prostate cancer, including prostate imaging, biopsy, and prostate-specific antigen (PSA) screening (Bryant et al. 2012; Prensner et al. 2012). However, prostate imaging and biopsy are difficult to use for early-stage diagnosis of prostate cancer. Thus, an *in vitro* detection method is necessary, since such a method has many advantages for early diagnosis of the disease (Singh et al. 2008). For early diagnosis of prostate cancer, a screening of PSA, a specific biomarker for prostate cancer, has been utilized. However, PSA screening is not a suitable method to distinguish between prostate cancer and other prostate diseases, such as benign prostatic hypertrophy (BPH), implying that PSA screening exhibits low sensitivity and specificity for early diagnosis (Ben Jemaa et al. 2010). Therefore, the development of an *in vitro* diagnostic method based on a stronger biomarker than PSA has been strongly demanded.

Transcription factors can be utilized as biomarkers to diagnose diseases because the abnormal expression of transcription factors leads to a dysregulation in the transcription of DNA to messenger RNA, finally resulting in the induction of various diseases, such as cancer and inflammation. Among the several transcription factors, engrailed-2 (EN2) is essential, as it contains the homeobox domain, which plays a key role in early embryonic development (Morgan 2006). EN2 is well known as a potential biomarker for several cancers, including breast cancer, ovarian cancer, bladder cancer, and prostate cancer (Martin et al. 2005; McGrath et al. 2013; Morgan et al. 2013). In particular, it has been reported that the expression level of EN2 increases in prostate cancer tissue and that urinary EN2 is more

specific and sensitive to prostate cancer than PSA, with a specificity of 88.2% and a sensitivity of 66% (Morgan et al. 2011a). In addition, urine samples can be abundantly obtained in a non-invasive manner to allow for *in vitro* diagnosis. Therefore, urinary EN2 has been considered a strong potential biomarker for *in vitro* diagnosis of prostate cancer.

There are reports related to a detection system for EN2 using a typical enzyme-linked immunosorbent assay (ELISA) (Morgan et al. 2011a). However, ELISAs have a number of disadvantages, such as high costs for monoclonal antibodies, inaccuracy due to blocking failure, and lengthy procedures (Hu et al. 2014). On the contrary, electrochemical biosensors offer rapid, simple, and low-cost sensing systems (Kang et al. 2009; Min et al. 2008). In particular, electrochemical impedance spectroscopy (EIS) has been applied to monitor the interaction and reaction of biomolecules on the electrode surface because it provides sensitive and selective electrochemical signals (Li et al. 2007). Specifically, EIS can be used to detect various biomolecules, such as oligonucleotides (Sosnowska et al. 2013), proteins (Lee et al. 2012a), and even cells (Min et al. 2010), without any labelling.

In this study, we designed an ultrasensitive impedimetric biosensor for EN2 based on the interaction between homeodomain and specific DNA probes. The bindings of EN2-DNA probes were confirmed through various assays, including an electrophoresis mobility shift assay (EMSA), a surface plasmon resonance (SPR), and a fluorescence assay (Kong et al. 2013; Liu et al. 2013; Tan et al. 2012). Then, the electrochemical cell was fabricated based on the electrodeposition of gold nanoparticles onto the gold electrode surface. It is well known that the deposition of gold nanoparticles onto the electrode surface results in a large active surface for highly sensitive detection (Chen et al. 2011). Therefore, the electrodeposition of gold nanoparticles was introduced in this work to create just such a valuable detection platform. The quantitative detection of recombinant EN2 was performed using an electrochemical biosensor, and a very low detection limit was achieved. In addition, the

specificity of the biosensor was checked for several other proteins in an artificial urine medium (AUM) to verify the reliability and effectiveness of the sensing system.

2. Materials and methods

2.1. Materials

The engrailed homeobox 2 (EN2) gene was purchased from Thermo Scientific (USA). The pET-28a plasmid was purchased from Novagen (Germany). The BL21(DE3) *Escherichia coli* strain, the Dynabeads His-Tag Isolation and Pulldown, and SYBR Green I (SGI) were bought from Invitrogen (USA). The *i*-pfu polymerase was procured from iNtRON biotechnology (Korea). All oligonucleotides were synthesized from Bionics (Korea) and Cosmo Genetech (Korea).

2.2. Cloning, expression, and purification of EN2

The EN2 gene was amplified from the cDNA using the *i*-pfu polymerase with the forward primer *EN2F* (5'- CCC GGA TCC ATG GAG GAG AAT GAC CCC AAG C-3') and the reverse primer *EN2R* (5'- CCC CTC GAG CTA CTC GCT GTC CGA CTT GC-3'). The amplified gene was cloned into the pET-28a bacterial expression vector and transformed into BL21(DE3). The cells containing the plasmid were induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and grown at 37 °C for 4 h. The harvested cells were resuspended in a lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM β -mercaptoethanol, 10 mM imidazole, and 5% (v/v) glycerol) and lysed by sonication at 4 °C. The resulting supernatant was loaded onto a Hi-trap Ni-NTA affinity column (GE Healthcare, USA) and purified using a 10 to 400 mM imidazole gradient. For further purification, the

eluted solution was loaded onto a MonoQ anion exchange column (GE Healthcare, USA) and then applied to the Superdex 200 gel filtration column (GE Healthcare, USA), which was equilibrated with a storage buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1.4 mM β -mercaptoethanol, and 10% (v/v) glycerol). The purity of the EN2 protein was analyzed through a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. EMSA

Various concentrations (0, 0.5, 1.0, 2.0, and 5.0 μ M) of the EN2 protein and the 1.0 μ M double-stranded DNA (dsDNA) probe (Table S1) were mixed in a reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM β -mercaptoethanol), and the mixtures were then incubated at 37 °C for 30 min. The EN2-DNA complex was loaded onto a 6% PAGE tris-borate-EDTA (TBE) gel and separated at 100 V for 50 min. Afterward, the gel was stained with SGI and coomassie brilliant blue to enable visualization of the DNA probes and the EN2 protein, respectively.

2.4. SPR

The (His)₆-tagged EN2 protein was immobilized on a Ni²⁺-charged Sensor Chip NTA (GE Healthcare, USA) by injecting 10 μ L of a 100 nM sample with flow rate of 2 μ L/min at room temperature. After washing the sensor chip with a running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μ M EDTA, 0.005% Surfactant P20), the 500 nM DNA probe solution (180 μ L) in the running buffer was injected into the chip with a flow rate of 30 μ L/min for association, and the running buffer was injected for the dissociation of the DNA probe for 7 min. The dissociation of the probe was carried out for a period of time sufficient to examine

the strong interaction between the probe and the EN2. The SPR data were obtained using the Biacore 2000 system (GE Healthcare, USA).

2.5. Quantitative fluorescence assay

The 1.0 μ M hpDNA3 probe in the reaction buffer was heated at 90 °C for 3 min and cooled at 4 °C for 1 h. The DNA probe was incubated with various concentrations of the EN2 protein (0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, and 100 nM) in the reaction buffer at room temperature for 20 min. SGI (10,000 $\times$) was diluted to 50 $\times$ with dimethyl sulfoxide (DMSO) before use. The prepared SGI was added to each DNA-EN2 mixture solution to produce a final concentration of 0.15 $\times$. The mixtures were incubated at room temperature for 20 min. Then, the fluorescence emission spectra were obtained at an excitation wavelength of 497 nm using the QuantaMaster QM4 spectrofluorometer (PTI, USA).

2.6. Determination of the dissociation constant (K_d)

Various concentrations (0, 1, 5, 10, 20, 50, 100, and 200 nM) of FAM-modified DNA probes were mixed with the solution containing EN2-immobilized magnetic beads (Cho et al. 2010). The mixtures were incubated through mild shaking at room temperature for 1 h (Lee et al. 2012). To remove any unbound DNA probes, the magnetic beads were separated from the mixture solution with an external magnet and rinsed with the reaction buffer containing 0.01% (v/v) tween 20 with mild shaking for 3 min. These washing procedures were repeated three times. Next, the DNA probe-EN2 complexes were eluted with 300 mM imidazole. The fluorescence intensity of the FAM-modified DNA probes was measured at 520 nm (λ_{exc} =494

nm) using a 1420 Victor multilabel counter (Perkin–Elmer, USA). Finally, the dissociation constant was calculated by fitting to the exponential decay 1 model of the Origin software.

2.7. Fabrication of electrochemical biosensor

A gold electrode was immersed in a piranha solution prepared by mixing sulfuric acid and hydrogen peroxide in a volume ratio of 3:1 at 90 °C for 5 min to clean the surface of the gold electrode. Then, the electrode was thoroughly rinsed with deionized water and dried using nitrogen gas. After that, the electrode was successively polished with 1, 0.3, and 0.05 μm alumina powders and cleaned by sonication in 50% ethanol. The electrochemical cell consisted of the gold electrode as the working electrode, an Ag/AgCl reference electrode in a saturated KCl solution, and a platinum counter-electrode. Electrochemical cleaning was performed by cycling the electrode potential between +0.1 and +1.6 V at a 50 mV/s scan rate in 500 mM sulfuric acid until a stable cyclic voltammogram was achieved. The cleaned electrode was immersed in a solution containing 6 mM HAuCl_4 and 100 mM KNO_3 . Electrodeposition of the gold nanoparticles onto the surface of the electrode was performed by cycling the electrode potential between +0.1 and +1.6 V at a 50 mV/s scan rate for 20 cycles. Immobilization of the 0.5 μM poly(A)₁₀-hpDNA3 probe and the 0.5 μM 6-mercapto-1-hexanol onto the surface of the electrode was performed in phosphate-buffered saline (PBS) (pH 7.4) with mild shaking at room temperature for 16 h (Lee et al. 2012). Modification of the electrode was monitored by cyclic voltammetry (CV) and EIS measurements, which were performed in 10 mL of PBS containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Impedance spectra were recorded within the frequency range of 10 kHz to 100 MHz. All electrochemical assays were performed using a PARSTAT 2263 (Princeton Applied Research, USA).

2.8. Electrochemical detection of EN2

The electrochemical detection of EN2 was carried out by immersing the functionalized gold electrode in PBS containing various concentrations of the EN2 protein (10 fM, 100 fM, 1 pM, 10 pM, 100 pM, and 1 nM) with mild shaking at room temperature for 1 h (Lee et al. 2012). Next, the interaction between EN2 proteins and DNA probes on the electrode was monitored by EIS assay. Impedance measurements were performed in 10 mL of PBS containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$, and all spectra were recorded within the frequency range of 10 kHz to 100 MHz.

2.9. Detection of EN2 in artificial urine samples

The AUM was prepared according to the methods described in the literature (Brooks and Keevil 1997). All proteins were mixed into the AUM until final concentrations of 10 pM were reached. Prior to the experiments, each urine sample was filtered with a 0.22- μm syringe filter. The poly(A)₁₀-hpDNA3 probe-immobilized gold electrode was immersed in the urine samples at room temperature for 1 h with mild shaking, in the same way as described for the previous measurement. After that, the impedance signals were measured using the same protocol described above.

3. Results and discussion

3.1. Homeodomain-specific DNA sequence recognition

It is well known that the EN2 protein, a transcription factor, strongly binds to a specific DNA sequence to regulate transcription (Ades and Sauer 1994). Based on this intrinsic property, the DNA probes were designed to employ the specific binding site (5'-TAATTA-3'), flanked by extra sequences for the stability of the DNA probes (Table S1). The recombinant EN2 protein was obtained and purified using a bacterial expression system and fast protein liquid chromatography, respectively. The interaction between the DNA probes and EN2 was analyzed using EMSA to confirm that the designed probes could bind to EN2. DNA probes generally bind to target proteins within 10 min; thus, each mixture was incubated for 30 min to ensure full binding. Then, the resulting mixtures were loaded onto the native gel and separated at 100 V for 50 min (Lee et al. 2012). The dsDNA probe was utilized in an EMSA assay, and the probe strongly bound to the EN2 protein (Fig. S1A).

The probe-EN2 recognition was also confirmed using SPR. After the probes were injected into the EN2-immobilized NTA chip for association, a steady decrease was observed in the dissociation phase (Fig. S1B), indicating the occurrence of a very slow dissociation between the probes and the target protein due to their strong binding. Thus, the DNA sequences were utilized as probes to detect the EN2 protein.

3.2. Quantitative fluorescence assay

A simple fluorescence assay was performed to investigate the feasibility of the DNA probes for the quantitative detection of EN2 (Kong et al. 2013; Liu et al. 2013; Tan et al. 2012). The scheme of the fluorescence assay was described in Fig. S2. In the absence of the

target protein, SGI can be easily intercalated into not only the extra sequences but also the specific binding sequences, resulting in a strong fluorescence signal. However, in the presence of the target protein, the specific binding site is blocked by the strong affinity between the DNA sequence and the target protein. Thus, SGI cannot be intercalated into the specific binding site, leading to a decrease in the fluorescence signal. Various concentrations of EN2 were applied to the solution containing a constant concentration of the dsDNA probe. Following the addition of SGI, fluorescence spectra were obtained. The fluorescence signals did not show any changes, even after 10 min, implying that the reaction was almost complete (data not shown). Therefore, each reaction mixture was incubated for 20 min. As the concentration of EN2 increased, the emission at 520 nm gradually decreased, and 1 nM of EN2 was successfully detected (Fig. S3). These results suggest that DNA probes can be utilized to detect the EN2 protein.

3.3. Affinity of the DNA probes to EN2

To design a suitable DNA probe, the affinity between the DNA probes and the EN2 protein was investigated by changing the extra sequences of the specific binding site. First, the influence of the hairpin structure on the affinity was examined using measurements of the dissociation constants of five sequences, including four hpDNA sequences with varying loop lengths and one dsDNA (no loop). The dissociation constants of each DNA probe were measured using FAM-labelled DNA probes and magnetic beads (Cho et al. 2010). The K_d values of the DNA probes were within the range of 66.33 to 98.84 nM, with the lowest being the K_d value of the hpDNA3 (Fig. S4 and Table S1). These findings indicate that an 8-base loop is a supportive sequence for binding the target protein. In addition, the effects of the number of extra sequences in the stem part were investigated using the hpDNA with the 8-

base loop, and there was no significant difference in K_d values (Fig. S4 and Table S1). These results imply that the extra sequences in the stem part do not affect the affinity of the DNA probes.

3.4. Fabrication and confirmation of the electrochemical sensor

EIS has been utilized to monitor interaction events when immobilized probes on an electrode surface interact with a small quantity of target molecules in a solution. The formation of the complex on the electrode surface interrupts electron transport between the solution and the electrode, resulting in an increased resistance at the electrode surface. To improve the sensitivity of electrochemical sensors in various studies, many methods for modifying the electrode have been developed. Among these methods, the deposition of gold nanoparticles onto the electrode surface has been considered an outstanding technique due to its large active surface, biocompatibility, high conductivity, and convenience of immobilization for thiol-modified biomolecules. In particular, the electrodeposition of gold nanoparticles is widely utilized in EIS studies because the synthesis and deposition of particles can be carried out simultaneously (Chen et al. 2011).

[Fig. 1]

In this work, the gold nanoparticle-deposited electrode was fabricated using an electrodeposition method, as shown in Fig. 1. The functionalization processes for the gold electrode, including polishing, washing, electrodeposition of gold nanoparticles, and immobilization of DNA probes, were monitored by CV and EIS (Fig. 2). After the deposition

of the gold nanoparticles onto the gold electrode, the oxidation and reduction peaks were measured and found to display a slight increase in the CV spectrum (Fig. 2A). In addition, the charge transfer resistance (R_{ct}) values in the Nyquist plot decreased in comparison to those of the bare electrode (Fig. 2B). R_{ct} denotes the diameters of the semicircles in the Nyquist plots, which represents the resistance for transferring the electrons of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions. These values were obtained using PowerSuite software (ver. 2.55, Princeton Applied Reserch). The linear graph was constructed using R_{ct} values measured by the PowerSuite software. We expatiated on this R_{ct} value. These findings imply that the electron transport to the gold nanoparticle-deposited electrode increased because the active area of the working electrode was extended by the gold nanoparticles. DNA probes were immobilized on the electrode with 6-mercapto-1-hexanol, which enhances the binding of EN2 by offering sufficient room for the interaction. After the immobilization of the DNA probes, the oxidation and reduction peaks significantly decreased, and the R_{ct} values dramatically increased (Fig. 2). These results indicate that the resistance on the electrode surface was enhanced by the DNA probes. Similar patterns were observed when the EN2 protein was added to the sensing system. In addition, the merit of the gold nanoparticles-deposited electrode was demonstrated via comparison with the bare gold electrode. Similar experiments to those described above were performed with the bare electrode. As represented in Fig. S5, the gold nanoparticles-deposited electrode exhibited lower impedance values than those of the bare electrode. Furthermore, there was a significant increase in the impedance value in the case of the particle-deposited electrode when EN2 protein bound to each electrode, indicating that the gold nanoparticle deposition made the detection more sensitive.

[Fig. 2]

3.5. Optimization of the sensing conditions

To optimize the sensing conditions, the R_{ct} values were measured in the presence of the EN2 protein using various DNA probes. Even though the loop and stem parts of the DNA probes were optimized through the previous magnetic-bead-based assay, we performed the probe modification again to enhance the sensitivity and selectivity of the electrochemical sensor. To optimize the loop length, the DNA probes with 0 to 10 bases at the loop position were immobilized onto the gold nanoparticle-deposited working electrode. Following the addition of an identical concentration of the EN2 protein, the changes in the R_{ct} values were recorded. R_{ct} (relative, %) stands for the relative increases in R_{ct} values, compared to the DNA probe-immobilized electrode. The maximum increase in the R_{ct} was obtained in the DNA probes with the 8-base loop case, which had the lowest K_d values (Fig. 3 and Table S1). Furthermore, an appropriate stem length was also tested using a procedure identical to the one used above. When the DNA probe with a 10-base-pair stem was used, the R_{ct} values were lower than those of DNA probes with 14-base-pair or 20-base-pair stem cases (Fig. 3). These findings indicate that there must be proper space between the gold nanoparticle surface and the specific binding site for the DNA probe to efficiently interact with the EN2 protein. Therefore, linker bases were introduced in the DNA probes to enhance the sensitivity of the sensor. We examined the influence of the lengths of the linkers, using poly(A) linker lengths of 0, 5, 10, and 15 bases. The highest increase in the R_{ct} appeared in the case of the poly(A) linker length of 10 bases, implying that this length provided adequate room for the binding of EN2 (Fig. 3). These results suggest that the DNA probe for EN2 requires not only strong affinity to the target but also suitable space for binding. Therefore, the poly(A)₁₀-hpDNA3 probe (the hpDNA3 probe with the poly(A) linker length of 10 bases) was finally chosen as the probe molecule of the electrochemical biosensor for the EN2 protein (Fig. 1).

[Fig. 3]

3.6. *Electrochemical detection of recombinant EN2*

The interaction between the DNA probe and the EN2 protein was monitored using the electrochemical biosensor fabricated above. Various concentrations of EN2 (10 fM–1 nM) were incubated with the poly(A)₁₀-hpDNA3 probe-immobilized electrode. The R_{ct} values of the Nyquist plot increased in proportion to the amounts of the EN2 protein (Fig. 4), and the calculated detection limit was 5.62 fM. This proposed biosensor displayed very high sensitivity, which resulted from a combination of the high affinity of the probe and the efficiency of the electrodeposition of the gold nanoparticle method. Therefore, this electrochemical biosensor is readily applicable for quantitatively detecting the EN2 protein with high sensitivity.

[Fig. 4]

3.7. *Specificity and applicability of the biosensor*

In the previous work, it has been shown that EN2 in urine is highly predictive of prostate cancer, with a sensitivity of 66% and a specificity of 88.2% (Morgan et al. 2011b). Given such a highly predictive biomarker, the proposed biosensor must be capable of distinguishing the existence of the EN2 protein from among other proteins for the accurate diagnosis of prostate cancer. In addition, the stability and applicability of the biosensor is

crucial for the detection of the EN2 protein in patient urine samples because human urine contains diverse biomolecules, bodily wastes, and ions that can interrupt the effective detection of the target proteins via non-specific binding. For this reason, the specificity and applicability of the sensing system were evaluated using AUM and several other proteins such as human serum albumin (HSA), bovine serum albumin (BSA), thrombin, lysozyme, and TATA-binding protein (TBP), which is another transcription factor for the TATA box. The changes in the R_{ct} value in AUM were recorded every 5 min for 2 h to examine the stability of the biosensor in AUM, and there were no significant changes in R_{ct} values as time passed (Fig. 5). In addition, the impedance signals for each protein sample and AUM as a negative control were measured using the protocol outlined above to demonstrate the selectivity of the sensor. While the R_{ct} value for EN2 significantly increased, the R_{ct} values for the other proteins and AUM did not change (Fig. 6). Moreover, the impedance signal increased in the case of the mixture of all proteins used above, whereas the impedance signal was maintained in the case of the protein mixture without EN2 (Fig. 6). These results suggest that the developed biosensor using the homeodomain-specific DNA probe can be used as a powerful tool in the diagnosis of prostate cancer.

[Fig. 5]

[Fig. 6]

4. Conclusion

In the present study, DNA probes for EN2 were designed using the interaction between the homeodomain of the EN2 protein and the specific binding sequence. The strong affinity of the DNA probes to the target proteins was substantiated via EMSA, SPR, and quantitative fluorescence assays. To determine which structure was not advantageous for the detection of EN2, the dissociation constants of the DNA probes were determined using a magnetic bead-based assay. The results identified an 8-base loop as a proper sequence for binding the target protein. The electrochemical biosensor was developed based on EIS. The working electrode was modified by the electrodeposition of gold nanoparticles, which led to an increase in the sensitivity of the sensing system. The detection condition was optimized by monitoring the R_{ct} values for the DNA probes, leading to the eventual selection of the poly(A)₁₀-hpDNA3 probe. The EN2 protein was quantitatively detected by the biosensor with high sensitivity, and the detection limit was 5.62 fM. Moreover, the target protein was successfully detected in AUM, and the impedance signals for other proteins did not change. In conclusion, the proposed biosensor can be utilized to detect urinary EN2 for the diagnosis of prostate cancer. However, further steps, including clinical examinations using urine samples, must still be performed to validate the feasibility of the biosensor.

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Figure captions

Fig. 1. (A) Schematic illustration of the electrodeposition of gold nanoparticles and the detection of EN2 proteins. The small spheres represent the gold nanoparticles, and the helical lines represent the DNA probe. The EN2 proteins are illustrated as ribbon diagrams. (B) The poly(A)₁₀-hpDNA3 probe.

Fig. 2. The fabrication of the electrochemical biosensor. The deposition of gold nanoparticles onto the working electrode surface was performed via electrodeposition. The fabrication process of the electrochemical biosensor was monitored using (A) CV and (B) EIS.

Fig. 3. Optimization of the electrochemical biosensor. The experiments were conducted to find the proper DNA sequence. The R_{ct} values for various lengths of the loop (A), stem (B), and linker (C) were recorded. The error bars represent the standard deviations of three repeated measurements.

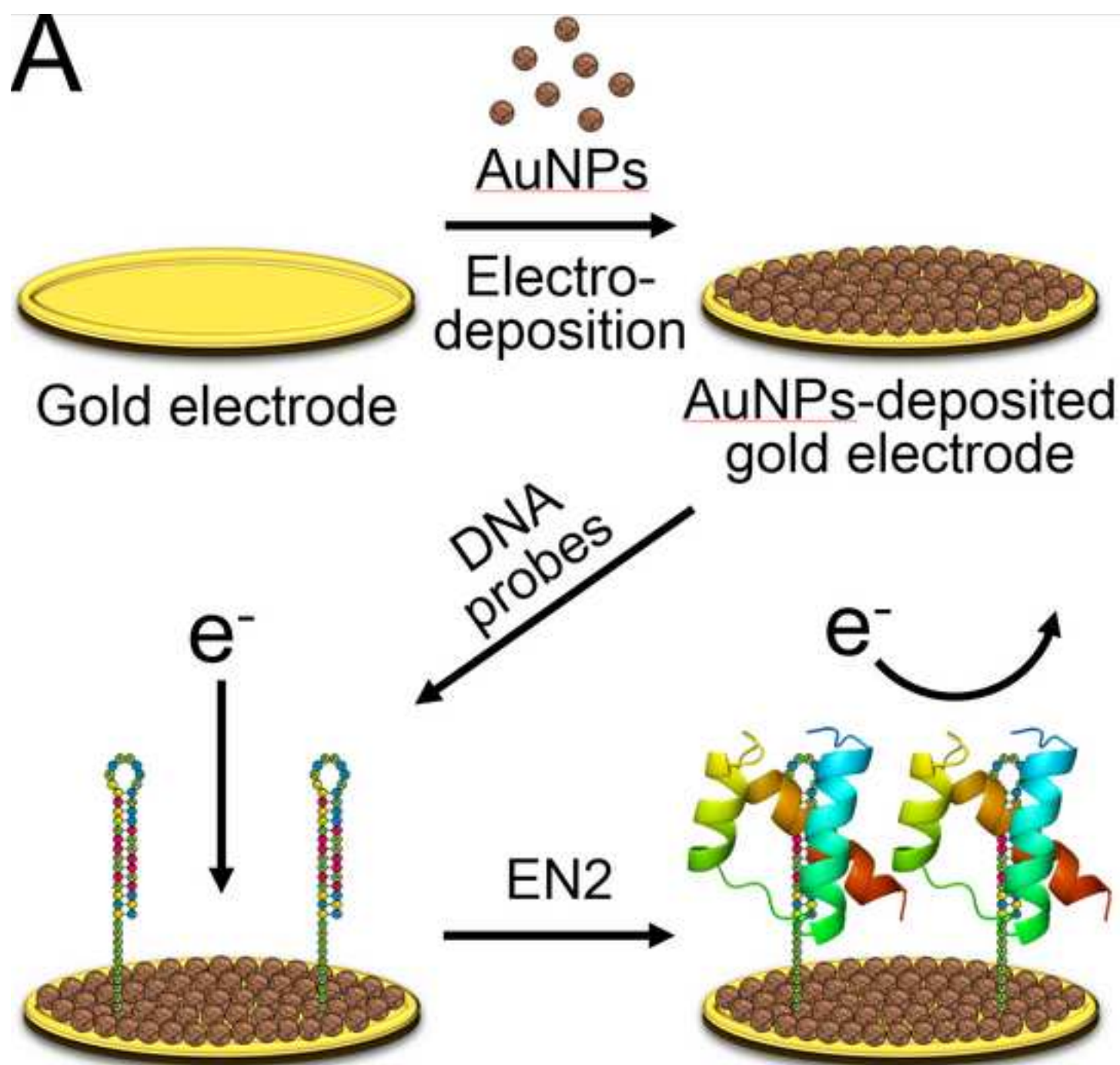
Fig. 4. Quantitative electrochemical detection of EN2. Nyquist plots for the electrochemical detection of EN2. Impedance values were measured for the DNA probe and EN2 at 10 fM–1 nM. The right plots represent the linear fit of the R_{ct} vs. $\log [EN2]$. The error bars represent the standard deviations of three repeated measurements.

Fig. 5. Stability of the biosensor. Impedance signals in the artificial urine medium were monitored over the course of time. The R_{ct} values were measured every 5 min.

Fig. 6. Specificity of the biosensor. Nyquist plots for the detection of several proteins in AUM. The R_{ct} values for AUM, HSA, BSA, thrombin, lysozyme, TBP, EN2, mixture without EN2, and mixture with EN2 are shown. The error bars represent the standard deviations of three repeated measurements.

Highlights

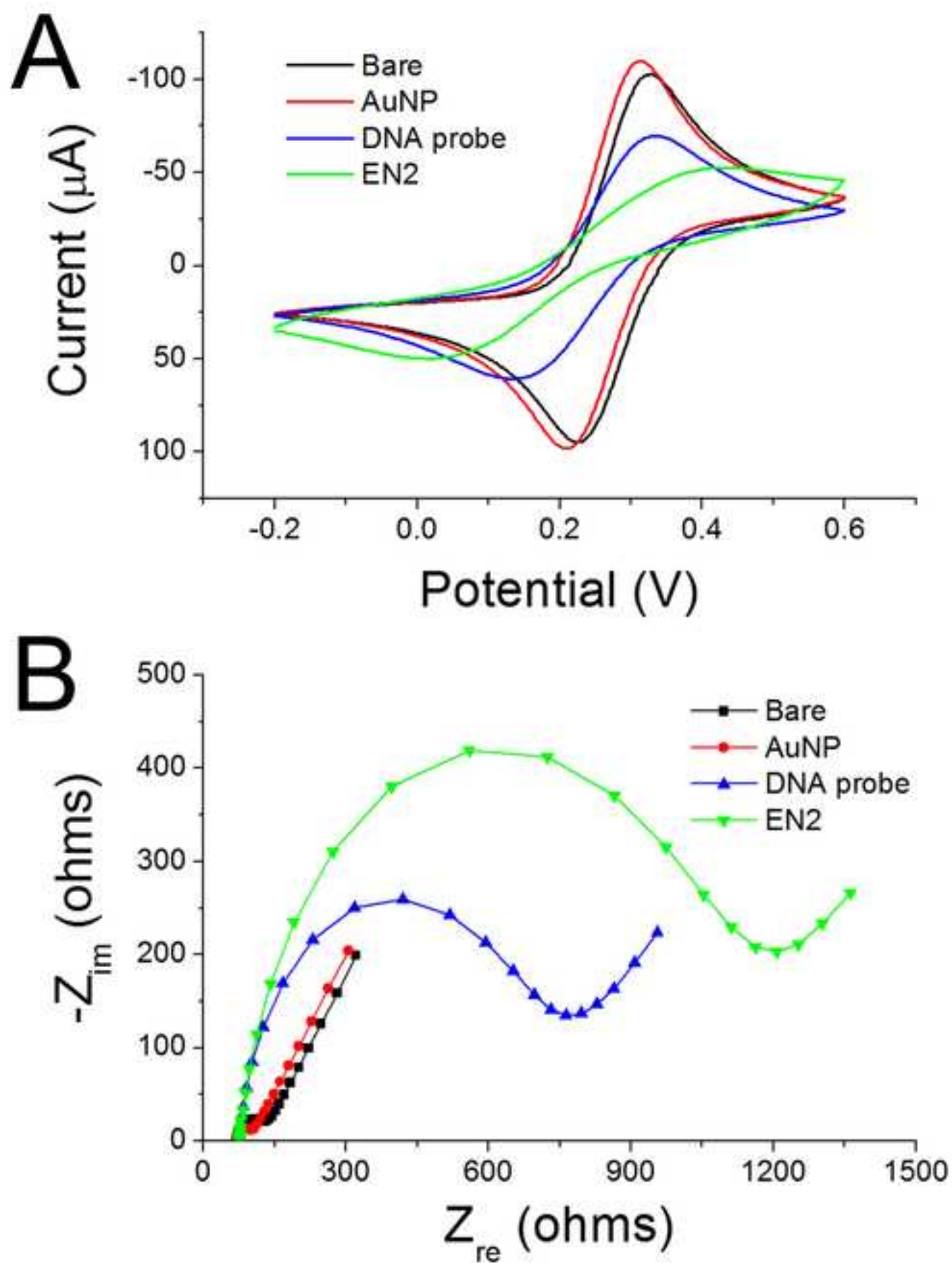
- The design of specific DNA probes for EN2 protein.
- The optimization of sequences via various techniques.
- The development of ultrasensitive electrochemical biosensor for EN2.
- Successful detection of EN2 with the detection limit of 5.62 fM.
- Valuable platform for early diagnosis of prostate cancer.



B

5'-thiol-poly A(10)-CGCA**TAATTA**CCTC^{C^AG_A}

3'-GCGT**ATTAAT**GGAG^{A_GG^A}



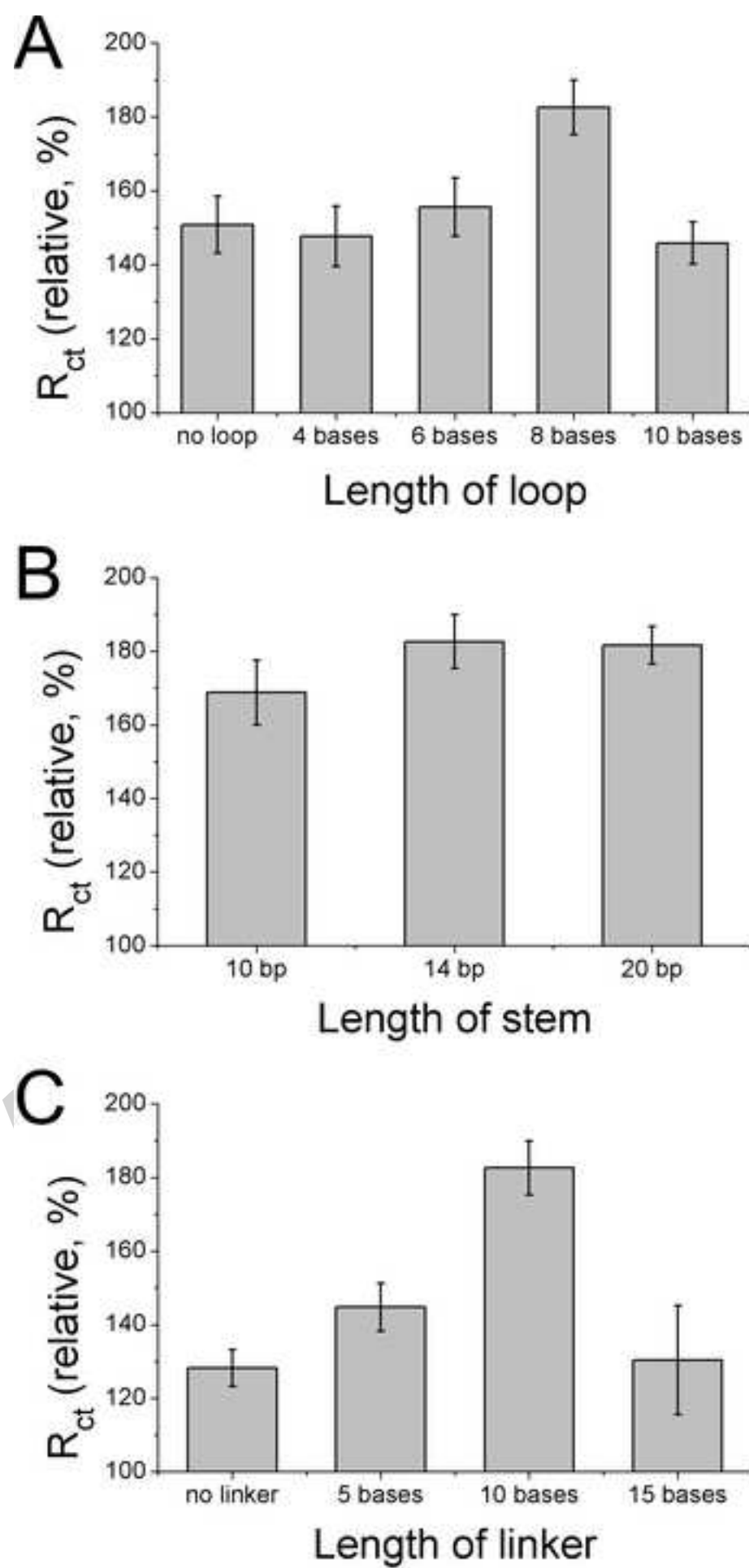


Figure 4

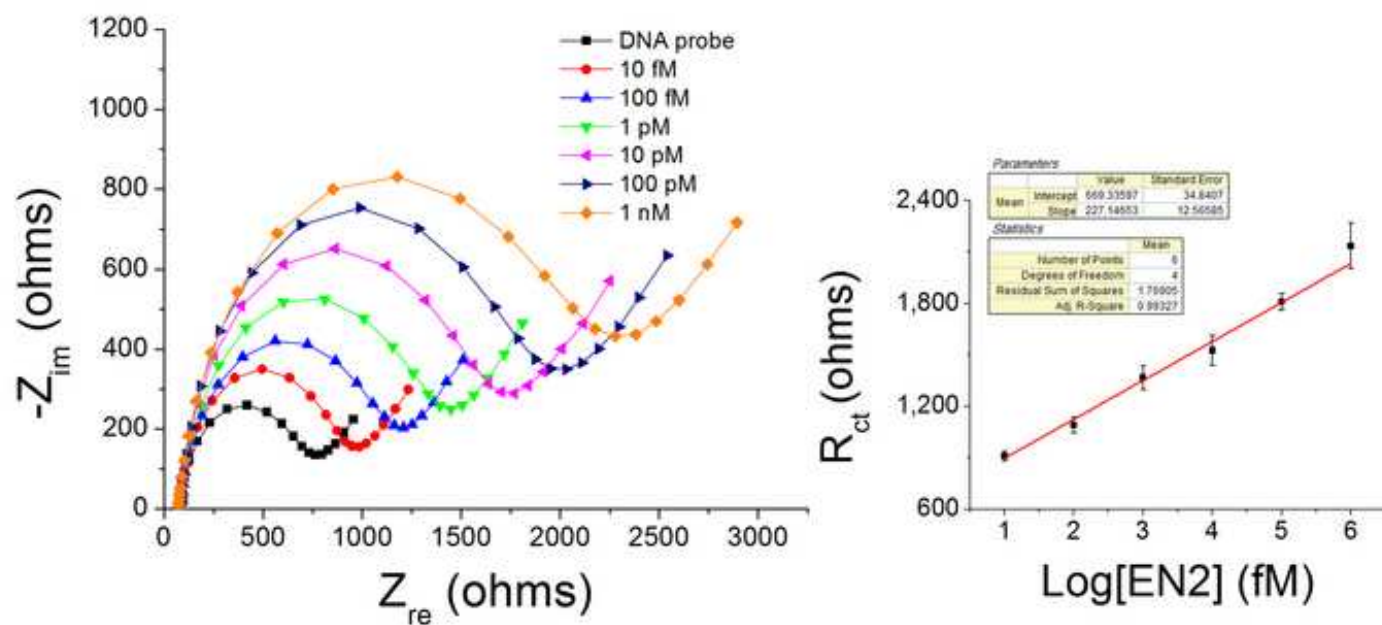


Figure 5

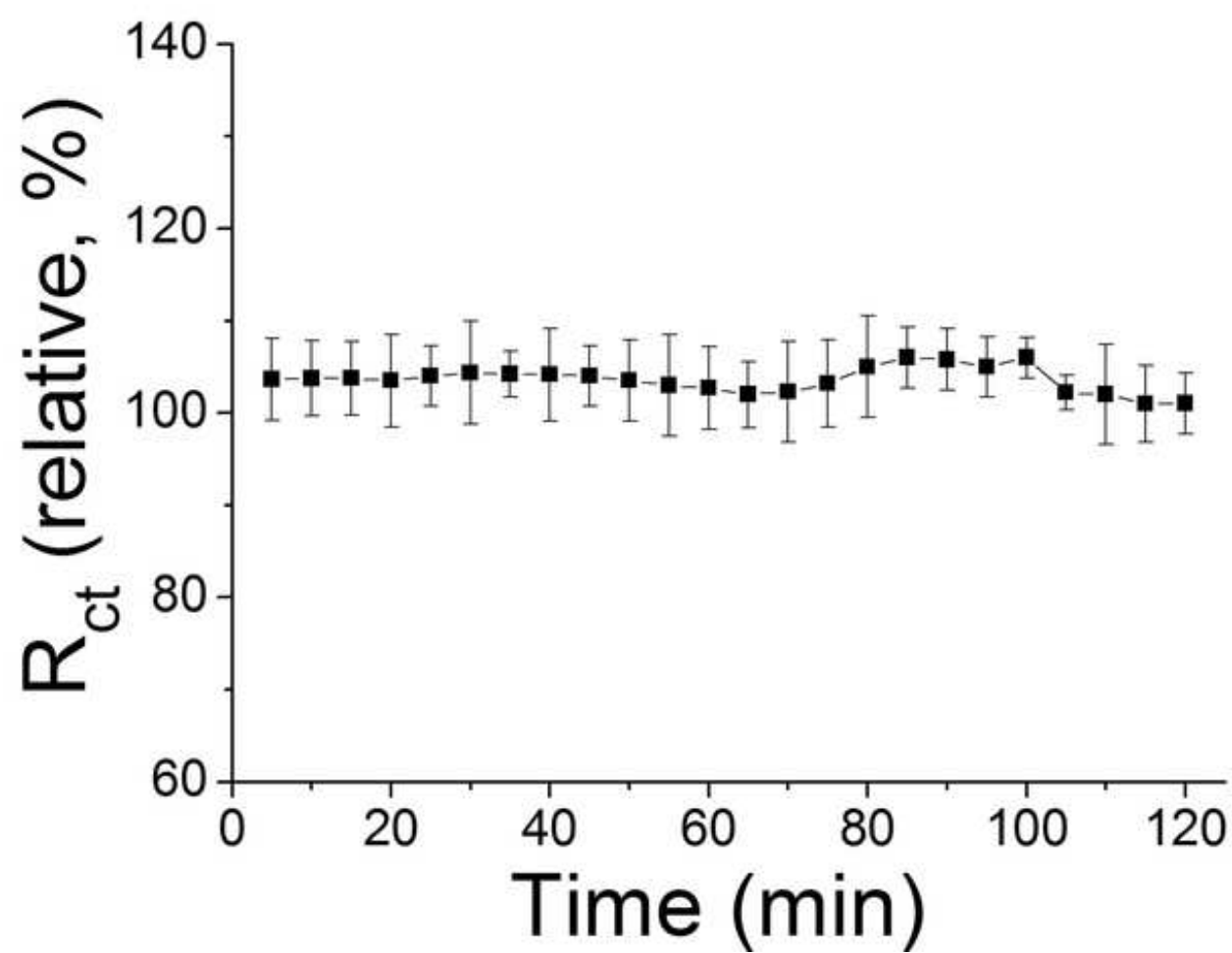


Figure 6

