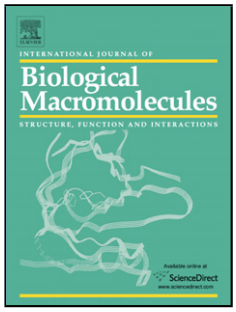

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SiO_2 nanoparticles modified CPE as a biosensor for determination of i-motif DNA / Tamoxifen interaction

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A B S T R A C T

Cytosine-rich DNA sequences can form a highly ordered structure known as i-motif in slightly acidic solutions. The stability of the folded i-motif structure is a good strategy to inhibit the telomerase reaction in cancer cells. The electrochemical biosensor was prepared by modifying carbon paste electrode with SiO_2 nanoparticles to investigate drugs which can stabilize this structure. Tamoxifen (Tam), an antiestrogen hormonal agent for treatment of breast cancer, was chosen as the model ligand and its interaction with i-motif structure was examined. The interaction between i-motif DNA and Tam was studied in PBS buffer and $[Fe(CN)_6]^{3-}$ through the cyclic voltammetry and square wave voltammetry methods. The oxidation peak of Tam, due to the i-motif DNA/Tam interaction, was observed after i-motif immobilized on the surface of the electrode. The i-motif formation was investigated by circular dichroism spectroscopy and the results showed that this structure can certainly be made with pH around 4.5, but its stability reduced by going to the more alkaline pH. The selectivity which was studied in the presence of complementary strand demonstrated that i-motif structure could be stabilized in acidic pH even in the presence of its complementary strand.

Keywords: i-motif DNA; Tamoxifen; Anti-cancer

1. Introduction

Telomeres, which were identified in 1938, are the protective DNA Protein complexes [1] that can protect the ends of eukaryotes chromosomes [2] and play an important role in cellular aging and cancer [3]. There is a telomere loss at every cell division that leads to cell cycle arrest and cell death. Telomerase, a special enzyme for telomeres sides, is responsible for maintaining the length of telomeres by synthesizing telomeric sequences [4]. This enzyme is activated in around 90% of cancers. in contrast to tumor cells, which often express high levels of telomerase [5], this enzyme is rarely expressed to significant levels in somatic cells [6]. Human telomeres are composed of tandem repeats of the double-stranded DNA sequence (5- TTAGGG): (5-CCCTAA) that may form a classical Watson-Crick double helix [7]. The oligonucleotides containing tracts cytosines can form a highly ordered structure known as I-motif structure at slightly acidic pH

[8]. The C-rich strand consists of four strands ordered in two parallel duplexes "zipped" together in an antiparallel direction, in which the C+C base pair form intercalation of two parallel-stranded duplexes [9].The stability of the i-motif depends on pH [10, 11], C-rich telomeric repeats, the nature and concentration of cations [7]. The stability of the folded i-motif structure at slightly acidic pH, provides a competitor for duplex formation relative to the linear C-rich strand at neutral pH, which results in slow hybridization occurring for duplex formation [12, 13]. The formed I-motif can act as a signpost and a controller for the oncogene expression at the replication level; thus, this structure has been a frequently encountered topic in nucleic acids research and as a promising material in nanotechnology [14, 15]. In this regards, finding ligands which can stabilize folded i-motif structure is a good strategy for cancer therapy; because it can be the inhibitor for duplex formation in telomere ends and induce a rebate at the telomerase reaction in cancer cells indirectly. Tamoxifen has been known as the most important hormonal agent for treatment of breast cancer for more than two decades. This drug can prevent approximately 50% of breast cancers in woman with increased breast cancer risk [16, 17].

Carbon paste electrodes (CPE) have gained greater importance in the field of electrochemistry due to their low residual current, less noise, easy preparation, their high sensitivity and renewable surface. These electrodes are also very economical [18-20]. Electrode surface modification is a field of paramount importance in modern electrochemistry especially due to the various potential applications of modified electrodes [21]. Oxide nanoparticles such as SiO_2, due to their large specific surface area and high surface free energy, have been extensively used for constructing electrochemical sensors and biosensors [22]. These materials are excellent subjects for immobilization of biomolecules according to their good biocompatibility and easy preparation [23]. Therefore, in this paper, CPE was modified with SiO_2 nanoparticles, and chemically modified CPEs were applied for the immobilization of i-motif DNA structure. The affinity of Tam as a selecting ligand to the i-motif structure was examined by using the electrochemical signal change and circular dichroism (CD) measurements.

2. Experimental

2.1. Reagents

Nanosilica with 12.6 nm average particle size was purchased from Nissan Chemical (Tokyo, Japan). The human telomeric DNA as probe and its complementary targets are d (5'-

CCCTAACCCTAACCCTAACCC-3') and d (5'- GGGTTAGGGTTAGGGTTAGGG-3'), respectively. The oligonucleotides were purchased from MWG-BIOTECH, Germany. The stock solution of DNA (1.0 μM) was prepared with phosphate buffered saline (PBS), (100mM phosphate, 1.5 M NaCl, 1.0 mM $MgCl_2$, pH 4.5) and was kept frozen. Double stranded DNA (dsDNA) formation was carried out by heating a solution containing ssDNA and its complementary sequences at 95 ˚C for 20 min in the presence of phosphate buffer (100 mM phosphate salt, 100 mM NaCl, pH 4.5 and pH 7.00) and followed by slow cooling to room temperature [24]. All other reagents were of analytical grade and purchased from Fluka or Merck. Water used for preparation of all solutions was deionized and distilled. All the electrochemical measurements were carried out at room temperature.

2.2. Instrumentation

Electrochemical experiments were carried out using an Autolab PGstut electrochemical analysis system, controlled by GPES 4.9 software (Eco Chemie Netherlands). Electrochemical studies were performed with a conventional three electrode system composed of a modified CPE as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl/KCl (3M) as the reference electrode. CD spectra were measured using an AVIV Model 215 CD spectrometer (Aviv, USA) from 320 to 200 nm. Standard quartz cells of 1 cm path-length were used for all CD experiments and the circular dichroism nerve network (CDNN) software was used for data analysis. The sample pH was adjusted for each experiment with HCl and NaOH.

2.3. Procedure

2.3.1. Preparation of modified carbon paste electrode

Carbon paste electrodes were prepared by mixing of graphite powder with paraffin oil in a ratio of 70:30 (w/w) in a ground homogeneously. The modified electrode was prepared by mixing graphite powder, L- cysteine and colloidal SiO_2 solution into the carbon before the addition of paraffin oil. A portion of the paste was then inserted in the bottom of a glass tube (i.d.5 mm). The electrical contact was established by a copper wire inserted through the opposite end in the inner hole of the tube. The surface of the resulting paste electrodes was polished on a weighing paper and rinsed carefully with distilled water to get a smooth surface. After each experiment, a

new surface could be produced by scraping out the old surface and replacing the carbon paste. For pretreatment of the polished electrode, potential was optimized at +1.80 V vs. Ag/AgCl/KCl (3M) for 5 mins in 0.50 M acetate buffer solution (pH 4.80) containing 100 mM of KCl without stirring [25]. All experiments were conducted on activated CPE and $NSiO_2$/CPE.

2.3.2. DNA probe immobilization on the $NSiO_2$/CPE

The i-motif DNA structure was immobilized on the modified CPE surface through physical absorption by applying positive potential to the electrode surface. In this regards, the modified CPE was immersed into an electrochemical cell containing i-motif DNA solution (100 mM phosphate buffer (pH 4.5), 1.5 M NaCl, 1 mM $MgCl_2$ and 1μM i-motif DNA) and the potential of +0.50 V vs. Ag/AgCl/KCl (3M) was applied to the electrode surface for 5 minutes. It was found that the immobilization of DNA probe on the SiO_2 modified electrode is a big challenge. This problem can be solved in some cases in the presence of Mg^{2+} [26], as adding $MgCl_2$ to the buffer solution improves the adhesion property of DNA on a high quality surface. Mg^{2+} ions attached on the negatively charged phosphorus in DNA can act as a bridge between DNA and SiO_2 nano particles [27, 28] but in some cases, even adding $MgCl_2$ cannot work, either. Here, cysteine was added to the CPE electrode material to solve this problem and improve the adhesion property of DNA on the electrode surface. As a result, in the presence of cysteine the resistance of SiO_2 nanoparticles decreased and DNA would immobilize better on this platform. The cysteine molecule undergoes an intermolecular charge interaction, inducing conformational changes due to protonation and deprotonating processes. When the pH value of the cysteine solution is lower than 5.02, the cysteine molecule will be positively charged and the positively charged interface in a phosphate buffer solution (pH 7.4) can be used as a binding force for further immobilization of negatively charged DNA from solution.

2.3.3. Interaction of i-motif with Tamoxifen on the surface of working electrode

To investigate the interaction of i-motif DNA with Tamoxifen on the working electrode surface, at first $NSiO_2$/CPE electrode was modified with i-motif DNA structure. Then, the immobilized i-motif/$NSiO_2$/CPE was immersed in the ligand solution and next, the SW voltammograms were recorded in the range of +0.8 to +1.2 V vs. Ag/AgCl/KCl (3 mol L^{-1}) at scan rate of 50 mV s^{-1} in buffer solution.

3. Results and discussion

3.1. Electrochemical characteristics of i-motif DNA/NSiO$_2$/CPE

3.1.1. Characterization of SiO$_2$ nanoparticles

Figure 1 contains SEM images of the CPE in different steps of electrode modification. Fig. 1A represents bare CPE and Fig. 1B indicates a relatively uniform distribution of the SiO_2 nanoparticles in the average diameter of 30-40 nm in size. Fig 1C shows the morphology of electrode after DNA immobilization. As can be seen, the NSiO$_2$/CPE surface changes to a compact structure in the presence of DNA. For investigation of the cysteine effect on the DNA immobilization, the SEM image was also detected in the absence of cysteine. As can be seen in Fig. 1D, no DNA could be immobilized on the surface in the absence of cysteine due to the resistance of SiO_2 nanoparticles.

Fig. 1

3.1.2. Cyclic voltammetry study on the immobilization of probe onto the modified electrode

The quality of the probe immobilization on the electrode surface was evaluated by cyclic voltammetry in 0.1 mol L^{-1} Phosphate buffer solution containing 0.01 mol L^{-1} $[Fe(CN)_6]^{3-}$ and 100 mmol L^{-1} NaCl, at a scan rate of 50 mV s^{-1} (Fig. 2). As it is shown in Fig. 2A, the intensity of $[Fe(CN)_6]^{3-}$ signal increased significantly after SiO_2 nanoparticles and cysteine deposition on the electrode surface (curve b). When ssDNA deposited on the modified CPE, the peak current decreased dramatically, indicating that the DNA was indeed immobilized on silica nanoparticles modified electrodes. The negatively charged phosphate backbone of the immobilized ssDNA repels the negatively charged redox probe $[Fe(CN)_6]^{3-}$. The results indicated that the immobilization of DNA on the modified CPE surface could greatly reduce the electron transfer rate of $[Fe(CN)_6]^{3-}$ due to the charge reputation between

DNA negativity phosphate groups and $[Fe(CN)_6]^{3-}$, but this behavior was not observed in the absence of cysteine (curve d).

3-2- Electrochemical behavior of i-motif DNA modified electrode in the presence of Tamoxifen

3.2.1. Cyclic voltammetry:

Fig. 2.B shows the interaction of Tam with the i-motif DNA modified electrode studied by cyclic voltammetry (CV) in the absence and presence of Tam in phosphate buffer (100 mM phosphate salt, 100 mM NaCl, pH 4.5) containing Tam. Fig. 2B shows the cyclic voltammograms of 1×10^{-5} M Tam at the surface of different electrodes. A well-defined oxidation peak at 1.07 V (curve a) could be seen in cyclic voltammetry for bare CPE. Curve b shows the cyclic voltammograms at the SiO_2/L-Cys modified CPE. The peak currents increased in comparison with curve a, which is due to the higher surface area of SiO_2 nano particles. Curve c shows the cyclic voltammetry after immobilization of i-motif DNA structure on the modified CPE. As can be seen, the peak current of Tam increased dramatically after i-motif DNA immobilization. It shows that after immobilization of i-motif DNA, Tam can intercalate in this structure such that its oxidation peak increased due to the Tam pre concentration on the electrode surface.

Fig. 2

3.2.2. Square wave voltammetry:

The square wave voltammetry (SW) was employed to ensure about i-motif DNA immobilization. This immobilization on the electrode surface was evaluated by SW measurement in 100 mM PBS buffer solution (pH 4.50). To investigate the interaction of i-motif DNA with Tam on the working electrode surface, i-motif DNA was immobilized at the modified CPE and then the immobilized i-motif DNA with modified CPE was immersed in ligand solution for 15 min without applying any potential to the working electrode. Then, the electrode was rinsed carefully with distilled water and SW measurements were recorded between +0.5 and +1.3 V vs. Ag/AgCl/KCl (3M) in PBS buffer. Fig. 3A (inset) shows the square wave voltammograms on the

bare CPE (a), SiO_2/CPE (b) and I-motif DNA/SiO_2/CPE (c). As it is clear, no peak was observed in the absence of Tam. Fig. 3A shows the i-motif modified electrode in the presence of different concentration of Tam from 7×10^{-8} to 1×10^{-5} mol L^{-1}. These results demonstrate that after the i-motif DNA deposition on the modified electrode surface, Tam can interact with this structure and its oxidation signal can be seen after this interaction. Fig. 3A shows that the oxidation signals of Tam increased by increasing its concentration (curve a-h). The calibration curve was obtained for constructed biosensor (Fig. 3B). The linear dynamic range and the detection limit from the calibration curve were 0.01-1 μM and 0.06 μM, respectively.

Fig. 3

In order to study the pH dependence of the proposed biosensor, the modified CPE was treated with i-Motif DNA structure in two different pH values (4.5 and 7). As can be seen in Fig 4A (curve a-b), the Tam oxidation peak appeared in pH = 4.5, which is related to the i-motif structure formation on the electrode surface which can interact with Tam through intercalation and stabilize this ligand on the surface. As can be seen in Fig 4A (curve c-d), no clear oxidation peak of Tam was observed in pH= 7.0 due to the less stability of this structure in this pH, which lead to no interaction with Tam and no oxidation peak observation.

To make sure about this discussion, this study has been conducted in the presence of double stranded DNA too. Fig. 4B shows SW voltammograms of Tam after immobilization of double stranded DNA on the surface of the electrode in two different pH (4.5 and 7.0). Fig 4B (curves a and b) shows the interaction between Tam and dsDNA in pH=4.5. The stability of dsDNA reduces in acidic pH, so i-motif structure can be formed and interact with Tam, which lead to the appearance of the Tam oxidation signal. By going to pH = 7.0, dsDNA become stable and obstruct i-motif formation; consequently, no obvious oxidation peak was observed for Tam in this pH range (curve c and d), which is due to the low stability of i-motif structure. The little peak, which was observed in more Tam concentration in the presence of dsDNA is due to the intercalation of Tam into dsDNA, which is very low in comparison with the one in i-motif structure.

Fig. 4

3.3. CD investigation of i-motif formation in solution

The distinguished characteristics of the i-motif structure can be studied by circular-dichroism (CD) spectra that are different from those of other DNA structures. To make sure about i-motif formation in the solution, CD experiment was used before using this structure to modify the electrode. CD spectra of i-motif DNA structure was recorded in 100 mM phosphate buffer (pH 4.5) containing 1.5 M NaCl, 1 mM $MgCl_2$ and was compared with the spectra in pH 7.40. The CD peaks show two typical characteristic peaks for i-motif structure, a positive band around 280 nm and a concomitant negative band around 260 nm [29]. Fig. 5 (curve a), illustrates the CD spectra of i-motif DNA structure. As it is clear, the CD spectra of i-motif showed a positive characteristic peak in 284 nm and a negative band around 266 nm, which confirmed the formation of this structure. The two characteristic bands shifted to less wavelength by shifting the pH towards 7.0 (curve b). It was concluded from the CD spectra that the i- motif structure can be formed in pH= 4.5, but its stability reduced by going to the alkaline pH.

Fig. 5

4. Conclusion

The absorption of biomolecules onto the surfaces of nanoparticles is important for biosensor construction. In this work the $NSiO_2$/cysteine was employed as a good platform for immobilization of DNA for construction of the i- motif biosensor. Tamoxifen has been introduced as the model ligand and its interaction with i-motif structure of human telomeric DNA sequences was examined. The I- motif formation and its interaction with the drug were studied according to the electrochemical signal and CD spectroscopy. It was found from the control experiment that the biosensor can distinguish i-motif structure from dsDNA and other single stranded DNA structure by changing the pH of the solution. The advantages of the proposed biosensor include rapid detection and the ability to distinguish i-motif DNA-binding ligands. The proposed biosensor in this study can be utilized for detection and discrimination of PCR products and plasmid of human telomeric DNA.

References

[1] R.W.L.C. Simon, H.B. Elizabeth, Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 359 (2004) 109-122.

[2] E.H. Blackburn, Cell, 106 (2001) 661-673.

[3] C.W. Greider, Proceedings of the National Academy of Sciences of the United States of America, 95 (1998) 90-92.

[4] L.G. Andrews, T.O. Tollefsbol, Methods in molecular biology (Clifton, N.J.), 405 (2008) 1-8.

[5] N. Kim, M. Piatyszek, K. Prowse, C. Harley, M. West, P. Ho, G. Coviello, W. Wright, S. Weinrich, J. Shay, Science, 266 (1994) 2011-2015.

[6] J.W. Shay, S. Bacchetti, European Journal of Cancer, 33 (1997) 787-791.

[7] A.T. Phan, J.-L. Mergny, Nucleic Acids Research, 30 (2002) 4618-4625.

[8] J. Zhou, C. Wei, G. Jia, X. Wang, Z. Feng, C. Li, Molecular BioSystems, 6 (2010) 580-586.

[9] P. Bucek, R. Gargallo, A. Kudrev, Analytica Chimica Acta, 683 (2010) 69-77.

[10] J.L. Leroy, M. Guéron, J.L. Mergny, C. Hélène, Nucleic Acids Research, 22 (1994) 1600-1606.

[11] J.-L. Mergny, L. Lacroix, X. Han, J.-L. Leroy, C. Helene, Journal of the American Chemical Society, 117 (1995) 8887-8898.

[12] W. Li, P. Wu, T. Ohmichi, N. Sugimoto, FEBS Letters, 526 (2002) 77-81.

[13] T.A. Brooks, S. Kendrick, L. Hurley, The FEBS journal, 277 (2010) 3459-3469.

[14] T.A. Brooks, L.H. Hurley, Nat Rev Cancer, 9 (2009) 849-861.

[15] S. Ahmed, A. Kintanar, E. Henderson, Nat Struct Mol Biol, 1 (1994) 83-88.

[16] M. Colozza, E. de Azambuja, F. Cardoso, C. Bernard, M.J. Piccart, The Oncologist, 11 (2006) 111-125.

[17] B. Fisher, J.P. Costantino, D.L. Wickerham, C.K. Redmond, M. Kavanah, W.M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieand, E. Tan-Chiu, L. Ford, N. Wolmark, o.N.S.A. Breast, B.P. Investigators, Journal of the National Cancer Institute, 90 (1998) 1371-1388.

[18] N.S. Lawrence, R.P. Deo, J. Wang, Analytical Chemistry, 76 (2004) 3735-3739.

[19] C.M.V.B. Almeida, B.F. Giannetti, Electrochemistry Communications, 4 (2002) 985-988.

[20] P. Tomčik, C.E. Banks, T.J. Davies, R.G. Compton, Analytical Chemistry, 76 (2003) 161-165.

[21] P.K. Brahman, R.A. Dar, S. Tiwari, K.S. Pitre, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 396 (2012) 8-15.

[22] X. Luo, A. Morrin, A.J. Killard, M.R. Smyth, Electroanalysis, 18 (2006) 319-326.

[23] D. Tang, R. Yuan, Y. Chai, Analytical Chemistry, 80 (2008) 1582-1588.

[24] S. Dhakal, Z. Yu, R. Konik, Y. Cui, D. Koirala, H. Mao, Biophysical Journal, 102 (2012) 2575-2584.

[25] M.S. Hejazi, J.-B. Raoof, R. Ojani, S.M. Golabi, E.H. Asl, Bioelectrochemistry, 78 (2010) 141-146.

[26] Y.S. Jo, Y. Lee, Y. Roh, Materials Science and Engineering: C, 23 (2003) 841-846.

[27] Y. Okahata, K. Ijiro, Y. Matsuzaki, Langmuir, 9 (1993) 19-21.

[28] T. Kanno, H. Tanaka, N. Miyoshi, T. Kawai, Japanese Journal of Applied Physics, 39 (2000) L269.

[29] F. Seela, Y. He, The Journal of Organic Chemistry, 68 (2002) 367-377.

Figure legend:

Fig. 1. SEM image of (A) bare CPE, (B) $NSiO_2$-Cys/CPE, (C) DNA/$NSiO_2$-Cys/CPE and (D) DNA/$NSiO_2$ /CPE

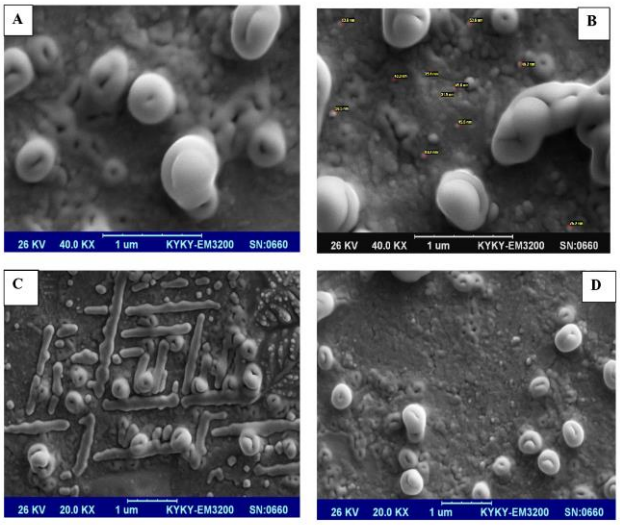
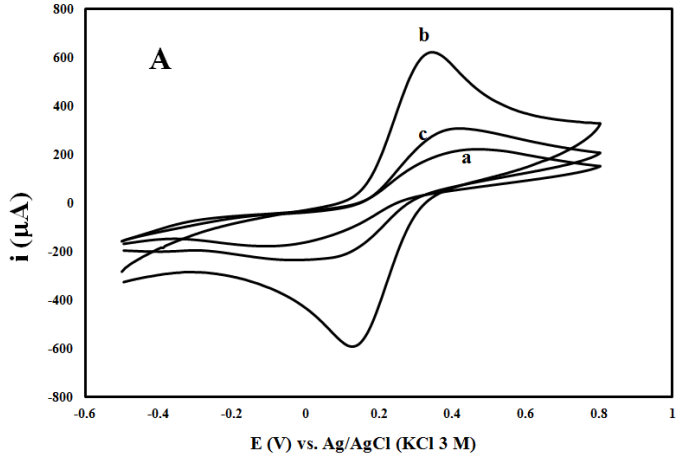


Fig. 2. Cyclic voltammograms of (a) CPE, (b) $NSiO_2$-Cys/CPE , (c) DNA/$NSiO_2$-Cys/CPE in 0.1 mol L^{-1} $[Fe(CN)_6]^{3-}$ and 100 mmol L^{-1} NaCl at a scan rate of 50 mV s^{-1}.**(B)** CV signals of 1×10^{-5} mol L^{-1} Tam in phosphate buffer (100 mM phosphate salt, 100 mM NaCl, pH 4.5) in the presence of CPE (a), $NSiO_2$-Cys/CPE (b) i-motif DNA/$NSiO_2$-Cys/CPE (c) at a scan rate of 50 mV s^{-1}.



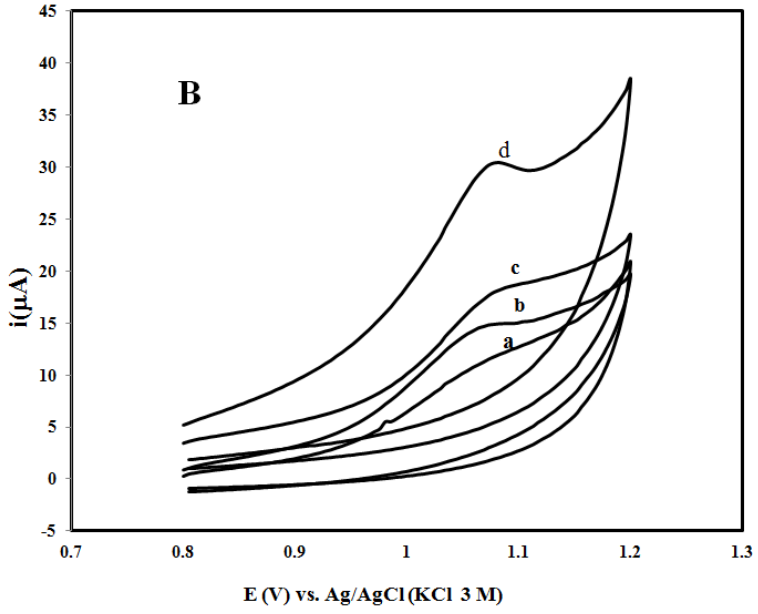
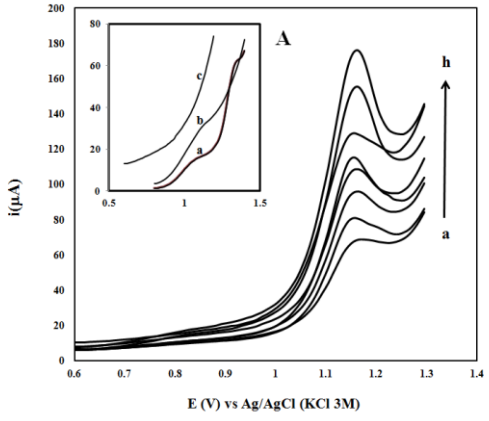


Fig. 3. (Inset A) Square wave voltammograms (SWVs) of CPE (a), SiO_2-Cys/CPE (b) DNA/SiO_2-Cys/CPE (c). **(A).** SWVs of DNA/SiO2-Cys/CPE in the presence of different concentrations of Tam on the surface of the electrode (pH 4.5): 7×10^{-8} (a), 1×10^{-7} (b), 5×10^{-7} (c), 7×10^{-7} (d), 1×10^{-6} (e), 5×10^{-6} (f), 7×10^{-6} (g), 1×10^{-5} mol L^{-1} (h) Tam in a 100 mmol L^{-1} PBS. **(B).** Saturation isotherm of Tam on DNA/SiO_2-Cys/CPE and calibration curves for Tam based on the linearized adsorption isotherm (Inset B), in PBS.



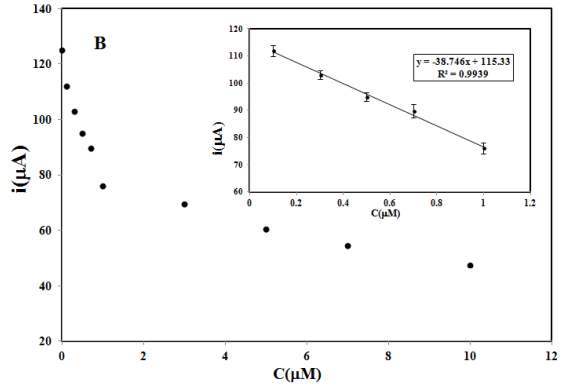
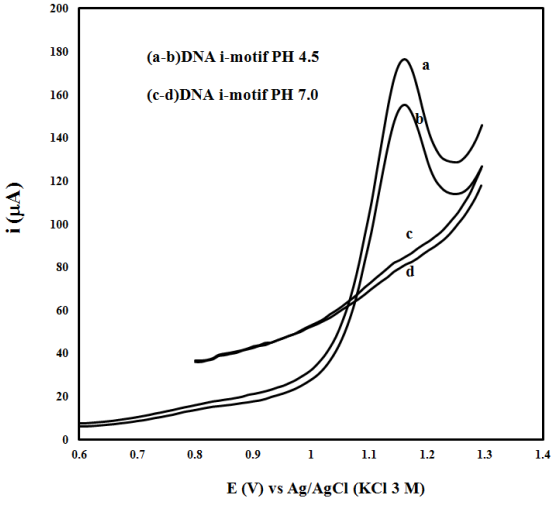


Fig. 4(A). SWVs of i-motif DNA/$NSiO_2$-Cys/CPE in the presence of 1×10^{-4} (a) and 1×10^{-5} mol L^{-1}(b) Tam in PBS buffer pH=4.5 and 1×10^{-4} (c) and 1×10^{-5} (d) mol L^{-1} Tam in PBS buffer pH=7.0 **(B).** SW voltammograms of dsDNA/$NSiO_2$-Cys/CPE in the presence of of 1×10^{-4} (a) and 1×10^{-5} (b) mol L^{-1} Tam in PBS buffer pH=7.0 and 1×10^{-4} (c) and1×10^{-5} (d) mol L^{-1} Tam in PBS buffer (pH=4.5) in PBS.



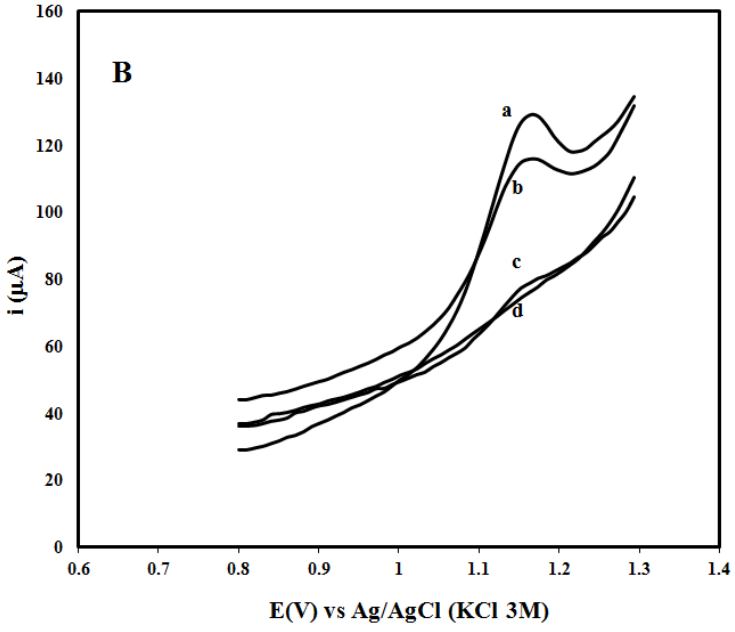


Fig. 5. CD spectrum of 1.0 μmol L^{-1} i-motif DNA in the pH=4.5 (a) and in the pH=7 (b).