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Detection and discrimination of recombinant plasmid encoding hepatitis C virus core/E1 gene based on PNA and double-stranded DNA hybridization

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ABSTRACT

Development of an electrochemical DNA biosensor for direct detection and discrimination of double-stranded plasmid (ds-Pl) without the need for denaturation of the target plasmid sample using a peptide nucleic acid (PNA) oligomer as the probe is described. This goal was achieved by modification of gold electrode with 6-mercapto-1-hexanol following monolayer self-assembly of cysteine conjugated 20-mer PNA oligomer probe, complementary to the HCV core/E1 region, which binds to ds-Pl and forms PNA/ds-Pl structure. The significant variation in differential pulse voltammetric response of methylene blue on the probe modified electrode upon contacting with complementary double-strand plasmid to form PNA/ds-Pl triplex structure is the principle of target plasmid detection. The results indicated that the reduction peak current was linear with the concentration of complementary strand in the range of 10–300 pg/μl with a detecion limit of 9.5 pg/μl.

1. Introduction

Hepatitis C virus (HCV), a small enveloped single-stranded RNA virus that belongs to the *Flaviridae* family, is one of the main causes of chronic hepatitis in developing countries. As reported by World Health Organization (WHO), it is estimated that about 170 million people are infected with HCV around the world. These infected individuals are the cause for most new infections. There is no Food and Drug Administration (FDA) approved prophylactic vaccine for HCV and the effective antiviral therapy, interferon and ribavirin, is not successful in all cases and is also extremely costly (Reichard et al., 1997).

One of the most conserved regions of the HCV genome that has less sequence diversity among various genotypes is the *core* gene of HCV and extends across different genotypes, making it an ideal candidate for protective DNA-based vaccine. The core protein of HCV is a multifunctional protein and involved in many viral and cellular processes (Majeau et al., 2004). Recombinant plasmids are present as double-stranded structures and used in most recombinant organisms and recombinant protein production procedures. There are many reports for development of electrochemical DNA hybridization biosensors related to detection of short oligonucleotide target DNA and the number of reported DNA biosensors for detection of DNA as recombinant plasmid is very limited (Hejazi et al., 2008). Considering that detection of plasmids in the corresponding procedures is important, development of simple methods for detection of plasmids to reduce the assay time and the number of required procedures is desired.

Peptide nucleic acid (PNA) is a natural mimic of DNA and can bind sequence specifically to double-stranded DNA (ds-DNA) in analogy to triplex forming oligonucleotides (Egholm et al., 1992; Mateo-Martí et al., 2007; Nielsen and Egholm, 1999). A few studies are reported for detection of target DNA in ds-DNA form (Kuhn et al., 2002; Baker et al., 2006; Pournaghi-Azar et al., 2010; Hejazi et al., 2011; Ananthanawat et al., 2011; Ahour et al., 2012). Following our previous studies about direct detection of ds-DNA target samples at oligonucleotide (Pournaghi-Azar et al., 2010; Ahour et al., 2012) and PCR product levels (Hejazi et al., 2011), we stepped forward to detect target sequence in longer DNA samples and herein direct detection of ds-DNA sample at plasmid level with 4568 bp long is reported.

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Here, for the first time, we describe an electrochemical approach for direct detection of taget DNA sequence in recombinant plasmid without denaturation of double-stranded DNA based on PNA probe and double-stranded DNA hybridization and using methylene blue (MB) as electroactive indicator.

2. Experimental

2.1. Chemicals

A cysteine conjugated 20-mer PNA chain (Cys-O-O-ATG TAC CCC ATG AGG TCG GC) was used as the probe. In this formula, HS-Cys represents the anchoring cysteine group and O is 8-amino-3, 6-dioxaoctanoic acid linker of the PNA chain. This probe contains a carboxamide ($CONH_2$) group at c-terminal. This sequence corresponds to a consensus sequence at the core/E1 region of HCV genome and consequently is used as a universal segment for detection of all HCV genotypes. Plasmids encoding this genomic consensus sequence are used for production of core/E1 recombinant protein and the produced protein in turn can be used for production of anti-HCV antibodies. Accordingly, development of fast methods for detection of plasmids encoding core/E1 sequence is of interest.

The PNA probe was dissolved in 0.1% trifluoroacetic acid and kept frozen at −20 °C. More diluted solutions of the PNA probe were prepared using 0.01 M Tris-HCl buffer solution (pH 7.0).

pBKC84 recombinant plasmid with 4568 bp length and containing core/E1 encoding cDNA (Kazemi et al., 2008) was used as a target plasmid and pET21a(+) plasmid was used as the non-complementary plasmid. More diluted solutions of the plasmids were prepared using 10 mM Tris–HCl buffer solution (pH 7.0). Other chemicals were of analytical reagent grade. The distilled, deionized and sterilized water was used in all solution preparations. All the experiments were performed at room temperature in an electrochemical cell.

2.1.1. Preparation of the working electrode

For preparing working electrode, gold disk electrode (7 mm^2 area) was polished with wet alumina slurry for at least 10 min and rinsed repeatedly with water. The polished electrode was then dipped in 1:1 EtOH–H_2O solution containing 0.5 M $NaBH_4$ for 10 min (Yuan et al., 2008). The electrode was treated with cycling the potential between −0.3 and 1.5 V with a scan rate of 0.1 V s^{-1} in 0.05 M H_2SO_4 solution until the cyclic voltammogram did not change, indicating that the electrode surface was clean. The electrode was then washed with water, and placed upside-down in a humidity chamber. A 6 μl droplet of 5 μM PNA probe was deposited on the pre-treated gold electrode for self-assembly. This probe self-assembled AuE was rinsed with water and then dipped in 1 mM aqueous solution of 6-mercapto-1-hexanol (MCH) at room temperature for 30 min. The post-treatment with MCH displaces non-specifically adsorbed PNA molecules and fills pinholes within the PNA monolayer. Evaluation of the PNA self-assembled monolayer (SAM) formation was performed using the electrochemical signal of the Fe $(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ redox system.

2.1.2. PNA probe and ds-DNA hybridization and labeling of the hybrid

The plasmid molecules were used for hybridization without denaturation as double-stranded structure. For PNA/ds-DNA hybridization, a 6 μl droplet of target ds-Pl in Tris–HCl buffer was deposited onto the electrode in the humidity chamber for 3 h. The same protocol was applied for interaction of the probe with non-complementary plasmid.

MB was interacted with the probe or the hybrid, by dipping the modified electrode surface in a stirring 20 mM Tris–HCl buffer (pH 7.0) solution, containing 20 μM MB with 20 mM NaCl, for 5 min without applying any potential. After accumulation of MB, the electrode was rinsed with 20 mM Tris–HCl buffer (pH 7.0) solution for 10 s.

2.1.3. Electrochemical measurements

The reduction signal of the accumulated MB was measured by using differential pulse voltammetry (DPV) in 20 mM Tris–HCl buffer (pH 7.0) solution containing 20 mM NaCl and scanning the electrode potential between −0.10 and −0.50 V at a pulse amplitude of 25 mV. The raw data were treated utilizing Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline correction using a 'peak width' of 0.01 between non-hybridized and hybridized status.

2.2. Instrumentation

All electrochemical experiments were performed in a 10 ml voltammetric cell, connected to an AUTOLAB PGSTAT 30 electrochemical analysis system and GPES 4.7 software package (Eco Chemie, the Netherlands). The utilized three-electrode system was consisted of a polycrystalline gold working electrode (Bioanalytical Systems, UK) with a 3 mm diameter, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. Supporting electrolyte was deoxygenated by purging nitrogen gas for 10 min prior to measurements, and the cell was blanketed with nitrogen gas during measurements.

3. Results and discussion

Target DNA detection strategies originally are based on analysis of DNA sample in single-stranded form (Degefa and Kwak, 2008; Ananthanawat et al., 2009; Wang et al., 1997; Meric et al., 2002; Hejazi et al., 2009, 2008; Pournaghi-Azar et al., 2008). This is achieved by denaturation of ds-DNA into ss-DNA. Denturation of ds-DNA into ss-DNA has some disadvantages such as follows: (a) denaturation is a further step and prolongs the detection time, (b) further steps means further contamination and (c) DNA usually is in double-stranded form in the nature and for this reason it is required to extract DNA from its natural sources and then denature ds-DNA into ss-DNA for detection purposes. Accordingly, development of strategies which rely on direct detection of ds-DNA will be of interest. A few studies are reported for detection of target DNA in ds-DNA form (Kuhn et al., 2002; Baker et al., 2006; Pournaghi-Azar et al., 2010; Hejazi et al., 2011; Ananthanawat et al., 2011; Ahour et al., 2012). By this, not only ds-DNA denaturation step is omitted, but also fundamental for detection of target DNA in real samples such as fixed samples is provided. Following our previous studies where we reported direct detection of ds-DNA target samples at oligonucleotide (with about 20 bp length) and PCR product levels (with about hundreds base pair length), we stepped forward to detect longer DNA samples and herein report direct detection of ds-DNA sample at plasmid level with 4568 bp long. The detection principle is based on the hybridization between the PNA and the target double-stranded plasmid. Plasmid molecules are present as double-stranded structures and are used in most recombinant organisms and recombinant protein production procedures. Considering that detection of plasmids in the corresponding procedures is necessary, development of novel detection methods which can reduce the assay time and the number of required procedures leading to simplification of the detection protocol and reduction in sample infection.

3.1. Preliminary investigation

Some experimental variables such as the probe SAM formation conditions (i.e., SAM formation time and probe concentration) affecting the performance of the sensor, were studied in the previous work (Ahour et al., 2012). Temperature has essential affect on the self-assembly formation and thus we studied this parameter effect on the probe formation. For this purpose the extent of the SAM formation on the electrode surface was evaluated by means of cyclic voltammetric measurement in $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox solution and on the basis of obtained results 25 °C was selected as the optimum temperature for electrode preparation (results not shown).

Detection of the hybridization was accomplished by monitoring difference between the DP voltammetric response of MB accumulated on the PNA–SAM modified AuE, before and after hybridization with complementary ds-pl. Fig. 1 shows the differential pulse voltammograms (DPVs) for the reduction signal of MB following self-assembly of the PNA probe onto the AuE (curve a) and after hybridization of the probe with the double-stranded complementary plasmid (curve b) as the target DNA. As seen in Fig. 1 significant increase in the MB signal (from -500 ± 6.1 nA to -1405 ± 35.36 nA for $n=5$) was occurred following hybridization of the PNA probe with the target plasmid (Fig. 1b). The observed increase in the peak current is addressed to the hybridization of the PNA probe with the target plasmid resulting in PNA/ds-DNA formation with which more MB molecules are interacted.

Having observed the ability of the electrode in detection of target DNA, an experiment with non-complementary ds-pl was carried out to assess whether the suggested DNA sensor responds selectively. For this purpose the non-complementary ds-pl was subjected to probe modified AuE. As seen in Fig. 1c the interaction between non-complementary ds-pl and immobilized probe also lead to a considerable increase in the MB reduction signal. This increase may be attributed to the adsorption of the sample impurities on the probe modified AuE or formation of partial unspecific hybrids between the probe and non-complementary ds-pl. In order to remove the adsorbed non-complementary ds-plasmid from the electrode surface or prevent its nonspecific immobilization on the probe-modified electrode some experimental variables were investigated and optimized.

3.2. Effect of treatment with RNase A enzyme

In the initial experiment we used plasmid samples without any purification. As we know there are considerable amount of RNA residues in the plasmid samples and these may be adsorbed on the electrode surface and disturb our experiment. Thus for this purpose plasmid samples treated with RNase A enzyme and purified plasmid samples were used for hybridization with self-assembled probe again. The obtained results (Fig. 2) showed that by using this treatment for plasmid samples, signal of the electrode after hybridization with non-complementary plasmid decrease while the signal of complementary one did not change. Thus we used RNase A treated samples for following experiments.

3.3. Effect of washing time and washing temperature

Washing time and washing temperature have essential effects on removing undesired oligomers from electrode surface. Subsequently for further decrease of the non-complementary signal and improving biosensor selectivity, we studied the effect of these

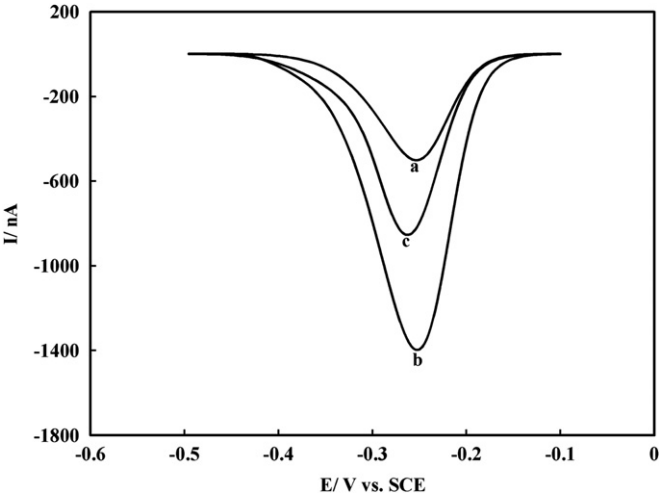

Fig. 2. Plot of MB peak current on the PNA probe modified AuE before hybridization (a) and after hybridization with RNaze A enzyme treated (b) complementary and (c) non-complementary plasmids. *Electrode conditions*: as mentioned in Fig. 1.

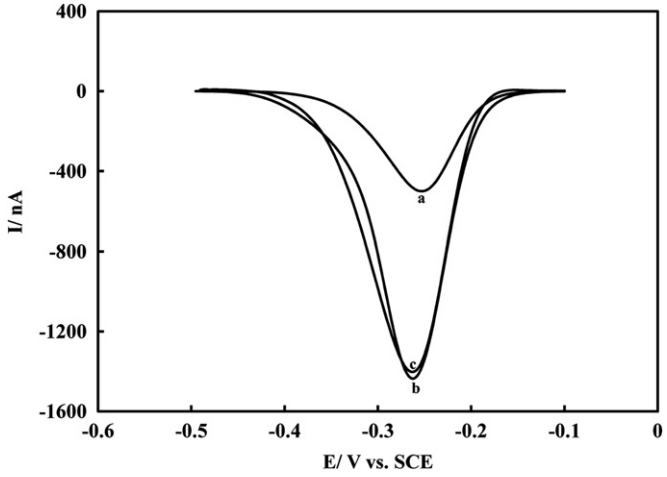

Fig. 1. Differential pulse voltammograms of accumulated MB on the PNA probe modified AuE: (a) before hybridization and after hybridization with 0.5 ng/μl, (b) complementary ds-pl and (c) non-complementary ds-pl. *Electrode conditions*: A 6 μl droplet of 1 μM PNA solution onto the pre-treated AuE for 2 h.

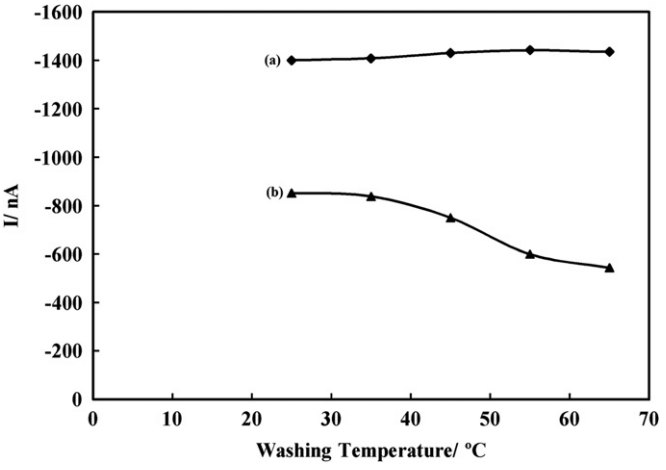

Fig. 3. Plot of differential pulse voltammetric signal of MB peak current on the PNA probe modified AuE after hybridization with 0.5 ng/μl (a) complementary ds-pl and (b) non-complementary ds-pl versus washing temprature. *Electrode conditions*: as mentioned in Fig. 1.

parameters on the hybridization signal. The obtained results showed that increasing the washing time up to 20 min has no effect on reduction of undesired signal (results not shown), but by increasing the washing temperature up to 55 °C (Fig. 3), signal of non-complementary plasmid decreases and selectivity of the biosensor increases.

Therefore, we used 5 min washing in Tris-HCl buffer by temperature 55 °C as the optimum condition for further studies.

3.4. Effect of plasmid DNA fragmentation

Our previous study showed that DNA fragmentation has many effects on the detection of genomic DNA samples and can be achieved by enzymatic digestion, sonication or vortex generated shearing forces. Here, we studied the effect of plasmid DNA fragmentation on the results. We used vigorous vortexing of the ds-plasmid samples to achieve fragmentation of large DNA molecules.

Fig. 4A represents the MB reduction signal of the self-assembled probe after hybridization with the non-vortexed and the vortex sheered plasmid DNA fragmentation. Results showed that there are no difference between the MB reduction signal of probe after hybridization with vortexed and non-vortexed samples. These findings showed that the vortex mediated fragmentation of the ds-plasmid DNA into small pieces has no effect on the hybridization efficiency and this is because of the small size of plasmid compared to genomic DNA. Thus, we used unvortexed plasmid samples for hybridization in further studies.

3.5. Effect of probe concentration

One important factor affecting the hybridization efficiency between the plasmid DNA and probe is the probe concentration during the probe film fabrication.

In this study, we used various concentrations of the probe in order to obtain an optimal PNA probe density for plasmid hybridization. The resulting MB peak currents showed that by increasing the probe concentration up to 0.5 μM, MB DP voltammetric signal is increased, but at higher PNA probe coverage (probe concentration greater than 2 μM), a decrease in the MB reduction signal was observed (Fig. 4B). We attribute this effect to a decrease in the number of triplexes formed on the electrode surface due to the steric and electrostatic hindrances arising from the more tightly packed PNA monolayer at higher surface coverage as described earlier (Herne and Tarlov, 1997; Peterson et al., 2001; Xu et al., 2008).

3.6. Effect of hybridization time

The influence of hybridization time on the hybridization efficiency was also studied and results are shown in Fig. 4C. As displayed in the inset of Fig. 4C, maximum amount of MB reduction signal was observed at 6 h hybridization time, but for saving in time and reducing the assay time, 3 h was selected as the optimum time for hybridization and this is enough for the present biosensor to act correctly, while the selectivity and sensitivity of determination remain nearly unchanged.

3.7. Selectivity of the method in optimized condition

The hybridization experiment with non-complementary oligonucleotide was carried out to assess whether the suggested DNA sensor responds selectively to the target. For this purpose, complementary ds-plasmid (pBKC84), and non-complementary ds-plasmid (pET21a) were subjected. As seen in Fig. 5 the interaction between pET21a and PNA–SAM modified AuE, did not lead to a significant increase in the MB reduction signal due to the absence of significant hybridization between the probe and non-complementary ds-plasmid (compare curve c with a). This result indicates that only the complementary ds-plasmid could form a PNA/ds-DNA structure and thus, the proposed biosensor can detect complementary plasmid and high selectivity of this new biosensor is confirmed.

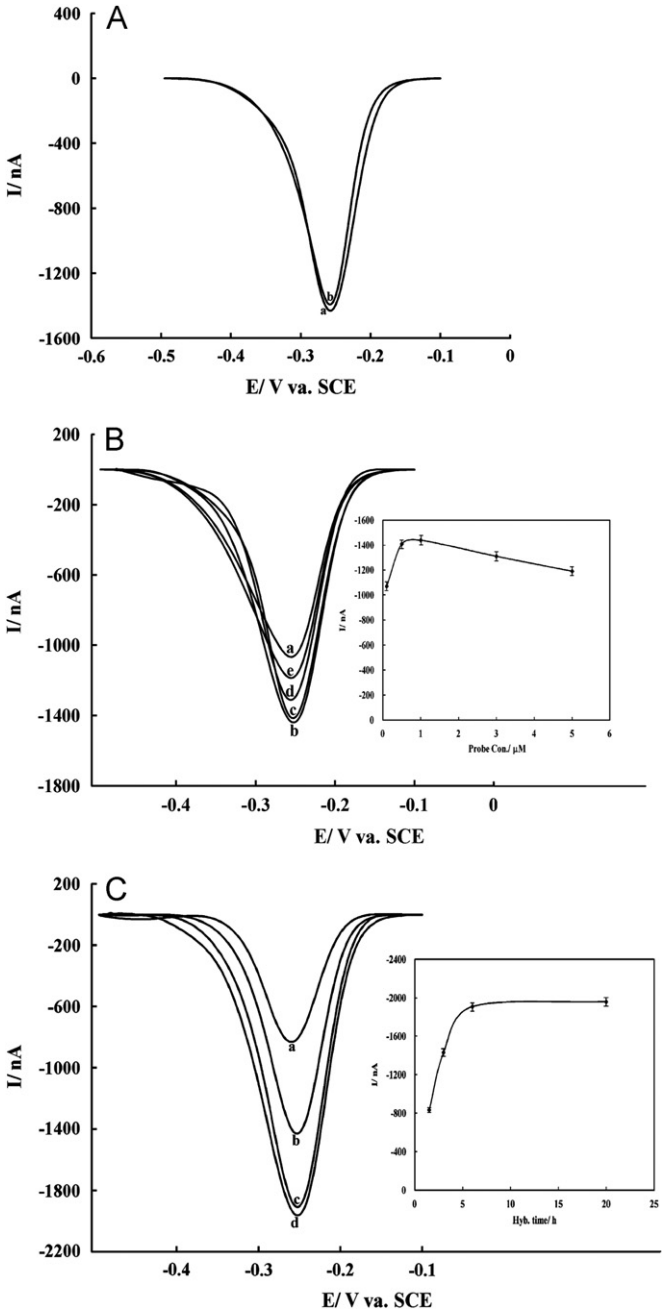

Fig. 4. Differential pulse voltammograms of accumulated MB on PNA probe-modified AuE (A) after hybridization with (a) vortexed and (b) non-vortexed complementary ds-pl samples; (B) using various probe concentrations, namely (a) 0.1, (b) 0.5, (c) 1, (d) 3 and (e) 5 μM, after hybridization with 0.5 ng/μl ds-pl solution. Inset variation of the MB peak current versus probe solution concentration; (C) after hybridization with 0.5 ng/μl of complementary ds-pl at various hybridization times: (a) 1.5, (b) 3, (c) 6, and (d) 20 h. Inset variation of the MB peak current versus hybridization time. *Electrode conditions*: as mentioned in Fig. 1.

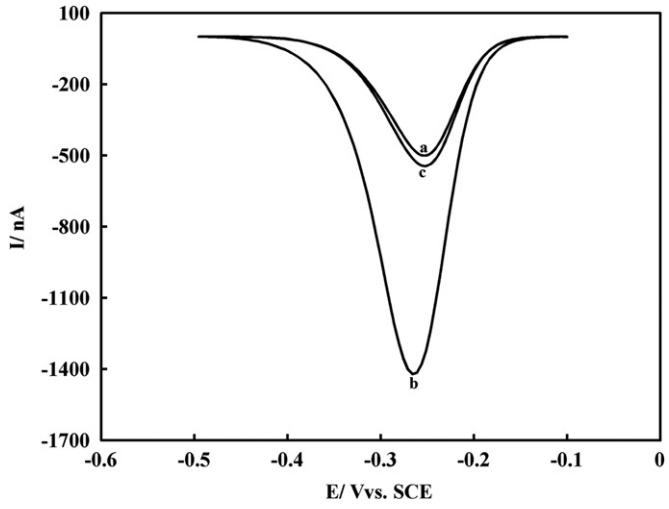

Fig. 5. Differential pulse voltammograms of accumulated MB on the PNA probe modified AuE: (a) before hybridization and after hybridization with 0.5 ng/μl (b) complementary ds-pl, and (c) non-complementary ds-pl. *Electrode conditions*: as mentioned in Fig. 1.

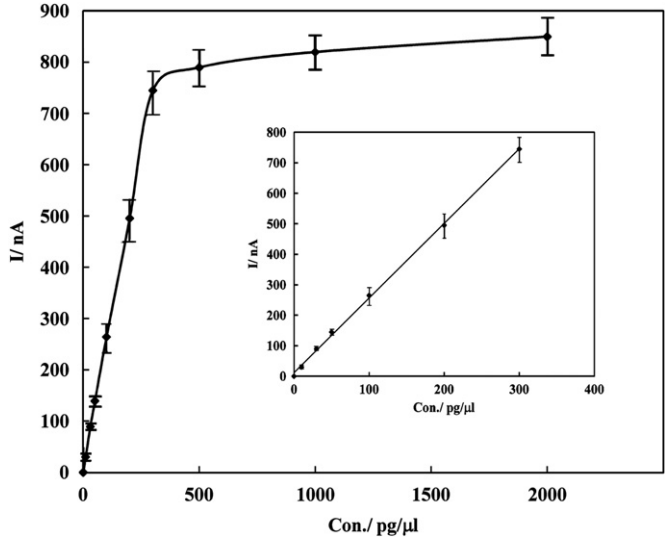

Fig. 6. Variation of the difference between the MB reduction signal of the PNA probe modified AuE in the presence and absence of complementary ds-pl (ΔI) versus target concentration. Inset of Fig. 6: related calibration graph at concentration range 10–300 pg/μl. *Electrode conditions*: as in Fig. 1.

3.8. Diagnostic performance

The difference between the signal of the MB accumulated on the PNA–SAM modified AuE in the presence and absence of the complementary ds-pl (ΔI) is increased with increasing concentration of the ds-plasmid, reaching a plateau at a concentration of about 500 ppm (Fig. 6). At this concentration of target ds-plasmid, the maximum capacity of the probe available on the electrode surface is involved in the hybridization. The calibration curve is linear between 10 and 300 ppm with a correlation coefficient of 0.999 for the complementary ds-pl. The calculated detection limit by means of equation: $y_{lod}=y_B+3S_{y/x}$ and regression equation: $\Delta I=-2.45C-10.08$ was about 9.5 ppm, where, y_B is the signal of blank (here intercept of calibration graph) and $S_{y/x}$ is the standard deviation of blank (here standard deviation of the calibration graph). The relative standard deviation in three independently probe modified electrodes (is obtained from measurements using three different electrodes) measured in the presence of 200 ppm complementary ds-pl was 3.7% (standard diviation is 40.2), indicating the reproducibility of the direct detection of the complementary ds-plasmid by this procedure.

4. Conclusion

Employment of PNA/ds-DNA hybridization for direct detection of longer ds-DNA samples at plasmid level was achieved. For this, a gold electrode modified with a self-assembled monolayer, composed of the PNA probe and MCH was employed for detection of core/E1 encoding cDNA of hepatitis C virus in double-stranded recombinant plasmid. The principle of the present strategy is based on the elevation of the MB peak current on the PNA probe modified electrode, due to the hybridization of the probe with the complementary ds-plasmid and maintenance of MB peak current of the modified electrode unchanged following interaction with non-complementary one. The increase in MB signal is related to PNA/ds-DNA hybrid formation and consequently more MB intercalation in the hybrid. The present strategy could be employed for development of further DNA biosensors for detection of ds-DNA in other ds-DNA samples without denaturation of the sample into ss-DNA.

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