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Investigation of Dilution Agent Effect onto Interactions Between Methylene Blue and DNA using Carbon Fiber Based DNA Biosensor

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Abstract: *This paper focuses on the dilution agent effect onto interactions between methylene blue (MB) and DNA using carbon fiber microelectrode (CFME) based DNA biosensor. In this study, designed CFME based DNA biosensor was successfully carried out considering the effect of probe dilution agent (e.g. 3-mercaptopropionic acid) against proposed hybridization mechanism types. The voltammetric signals of MB were measured at bare CFME, single-stranded DNA (ssDNA)-modified CFME and double-stranded DNA (dsDNA)-modified CFME by means of square wave voltammetry (SWV). The electrochemical parameters for MB on binding to DNA onto single CFME in the solution and at the electrode surface were described. This study shows that probe dilution agent is a significant factor in determining the type of DNA hybridization mechanisms.*

Keywords:

1. INTRODUCTION

The detection of specific DNA sequences has been of great interest for a long time due to its importance in many areas including clinical, food, biological warfare agent and environmental analysis. Studies about binding mechanism of redox-active molecules with DNA have been identified as one important topic to understand the mechanism of action or toxicity of different pollutants and drugs [1-3].

Electrochemical redox-active molecules capable of binding with different affinity to ssDNA and dsDNA are of particular interest for electrochemical analysis of DNA sequences [4,6]. It is widely known that MB, an aromatic heterocycle molecule, is often employed as an electrochemical redox indicator toward selective discrimination of ssDNA and dsDNA [3,6-16].

Former studies have indicated that MB binds to DNA through at least three different interactions; electrostatic interaction between cationic MB and anionic DNA, intercalation of MB in the DNA double helix and preferential binding between MB and guanine bases. Possibly due to the existence of such complicated interactions, various MB-based DNA sensing strategies have been suggested and some reports existing in the literature are even seemingly contradictory. Ozsoz et al. have reported ssDNA modified carbon electrodes produced large electrochemical signals for MB while hybridization led to considerable signal reduction [17]. In contrast, Ju et al. reported a signal-on DNA sensor that displayed an apparent increase of MB redox signals after DNA detection [18].

A lower MB reduction signal is observed upon DNA hybridization ascribed to the steric inhibition of the reducible groups of MB packed between the bulky double helix of the hybrid [19]. Alternative approaches are necessary to solve the contradictory results.

DNA immobilization on the transducer has an important role in the performance of the DNA biosensors. CFMEs can experience much higher current densities during electrochemical pretreatment that enhances electron transfer reactivity [20]. Because of its simple fabrication and high sensitivity, the CFME is widely used during the in vivo experiments [21]. Despite their many advantages for monitoring DNA and RNA in microliter samples, the use of carbon fibers for the analysis of nucleic acids has not yet been sufficiently reported.

In the literature, only after hybridization mechanism (probe+cDNA+MB) has been applied in the DNA biosensor studies containing MB as hybridization indicator [6-16]. However, different mechanisms are also possible and need to be investigated. Three different hybridization mechanism were developed between

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MB and DNA to obtain more specific and selective results. For this, MB was accumulated on ssDNA and dsDNA in three different ways: i) just before hybridization, ii) before and after hybridization, iii) only after the hybridization.

This paper focuses on the dilution agent effect onto interactions between methylene blue and DNA using carbon fiber based DNA biosensor. Various probe configurations were designed. The CFME-based biosensor was constructed by electrodeposition of gold nanoparticles and was investigated the effect of dilution agent with thiol-labeled ssDNA. The relationship between MB binding mechanism and designed probes was analyzed in detail. This study shows that probe with together dilution agent is more important to solve the MB binding problem.

2. EXPERIMENTAL

2.1. Materials

The synthetic oligonucleotides were obtained from Avetra Bioscience (Mountain View, CA, USA); their base sequences were:

- Thiol-labeled ssDNA probe (18-base):
  5'-SH-GCTGCCTCCCGTAGGAGT-3'
- Complementary target (18-base):
  5'-ACTCCTACGGGAGGCAGC-3'
- Noncomplementary target (18-base):
  5'-CTCTCTCTCTCTCTCTCT-3'

All oligonucleotides, dsDNA and ssDNA stock solutions (100 ppm) were prepared with TE solution (20 mM Tris-HCl, 100 mM EDTA, pH 8.00) and kept frozen.

Methylene blue (MB), tris(hidroksimetil)aminomethane and gold (III) chloride trihydrate were purchased from Sigma-Aldrich. 3-Mercaptopropionic acid (MPA) was obtained from Merck and reagents were all of the analytical reagent grade. All H_2O used in the preparation of buffers and for rinse solutions had a resistivity of 18.2 mΩ, as producted by Millipore Elix 5 UV and Milli-Q Gradient ultra-pure water system. Hybridization is carried out in the DNA hybridization buffer containing 50 mM Tris-EDTA-HCl and 100 mM NaCl, (pH 7.4, 25 ˚C). All the buffer solutions contained 20 mM NaCl.

High Strength (HS) carbon fibers C320000A (CA) (Sigri Carbon, Meitingen, Germany) containing 320,000 single filaments carbon were used to fabricate carbon fiber microelectrodes.

2.2. Instrumentation

An electrochemical measurement is performed on the electrochemical workstation (CHI 842B, CHI Instruments Inc., USA) in a typical three-electrode system with an Ag/AgCl electrode (saturated with KCl) as the reference electrode, a platinum wire as the counter electrode and a carbon fiber microelectrode (CFME) as the working electrode.

2.3. Experimental procedures

In this study, designed CFME based DNA biosensor was carried out considering the effect of probe dilution agent (e.g. 3 mercaptopropionic acid) against proposed hybridization mechanism types. Initially, MB was accumulated on ssDNA and dsDNA in three different ways; 1) before hybridization (probe+MB+cDNA), 2) before and after hybridization (probe+MB+cDNA+MB) and 3) after hybridization (probe+ cDNA+ MB) (Fig. 1). The most discriminative MB signals were investigated among the mechanisms.

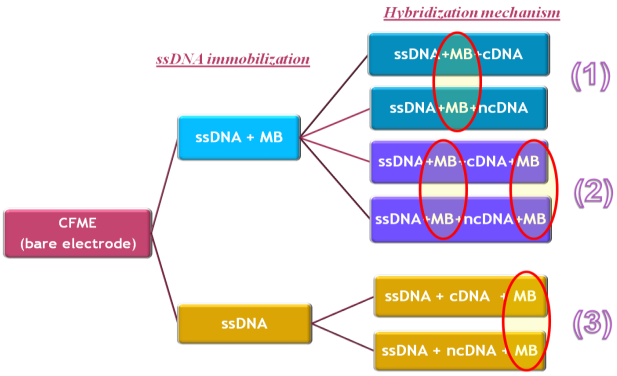

Figure 1. Process diagram for DNA biosensor according to the proposed hybridization mechanisms. MB was accumulated on ssDNA and dsDNA in three different ways; 1) before hybridization (probe+MB+cDNA), 2) before and after hybridization (probe+MB+cDNA+MB) and 3) after hybridization (probe+cDNA+MB).

Then, designed CFME based DNA biosensor was carried out considering the probe dilution agent effect onto MB binding mechanism to DNA according to the determination of the hybridization mechanism.

2.4. Preparation of CFME

All of the CFMEs were prepared by using single CFME (diameter ~7µm) attached to a copper wire with a Teflon tape. A half centimeter of the CFME was immersed into the solution to keep the electrode area constant (~ 0.0011 cm^2) and the rest of the electrode was covered with a Teflon tape [22].

2.5. Preparation of nano-Au modified CFME

The fabricated CFME was first consecutively sonicated in acetone, 3 M HNO_3, 1.0 M KOH, and distilled water each for 3 min. [23]. Then, the CFME was immersed in 0.2 mg/mL $HAuClO_4$ solution and was conditioned by cyclic sweeping from -0.8 to 0.8 V at 50 mV s^{-1} for deposition of Au nanoparticles. After twenty consecutive cycles, the electrode was taken out from the solution and rinsed with water; this is denoted as AuNPs/CFME [24].

2.6. Dilution of thiol-labeled ssDNA and immobilization of ssDNA on modified CFME

A self-assembled monolayer (SAM) was prepared by the immersion of the AuNPs deposited electrodes in freshly prepared immobilization buffer solution containing Probe:MPA ratio of 1:5 solution overnight for approximately 10 h, at 4 ˚C. Finally, the modified electrodes were rinsed with double distilled water thoroughly and obtained diluted thiol-labeled ssDNA probe with 3-mercaptopropionic acid (DPM).

2.7. Hybridization

For the DNA hybridization, ssDNA modified CFMEs were incubated with 10 ppm (µg/mL) complementary target DNA (cDNA) and noncomplementary target DNA (ncDNA) in the hybridization buffer at 47 ˚C or 120 min.

2.8. MB accumulation and voltammetric transduction

MB was firstly accumulated onto the ssDNA and the DNA hybrid coated electrode surfaces by immersing the electrode into the stirred 50 mM TRIS-HCl containing 20 µM MB for 5 min. The electrode was then transferred into the blank 50 mM Tris-EDTA HCl buffer solution (pH 7.4, 25 ˚C) with 100 mM NaCl for the voltammetric measurement. MB is a redox indicator with the formal potential in the range of 0 to -0.6 V. After accumulation of MB, electrochemical measurements were performed [25]. Triplicate measurements were carried out by renewing the surface and repeating the above assay preparation procedure.

3. RESULTS AND DISCUSSION

3.1. Investigation of MB signal between ssDNA and dsDNA to determine mechanism of MB binding

For thiol-labeled ssDNA (probe), the peak current density differences (ÄIp) were 0.32 mA/cm^2, 0.11 mA/cm^2, 0.30 mA/cm^2 before hybridization (probe+MB+cDNA), before and after hybridization (probe+MB+cDNA+MB) and after hybridization (probe+cDNA+MB), respectively (Fig. 2A). For thiol-labeled ssDNA probe diluted with MPA (DPM), the peak current density differences (ÄIp) were 0.13 mA/cm^2, 0.26 mA/cm^2, 0.02 mA/cm^2 for the three hybridization mechanisms, respectively (Fig. 2B).

As can be seen from Fig. 2A, comparing voltammetric MB peak current densities between thiol-labeled ssDNA (probe) and three hybridization mechanisms it is obvious that the first mechanism (probe+MB+cDNA) is the most discriminative. It was found that the peak current density of MB at ssDNA was considerably higher than at dsDNA. Whereas, according to Fig. 2B, the second mechanism (DPM+MB+cDNA+MB) should be preferred due to its discriminative peak difference with DPM. Contrary to Fig. 2A, it was found that the peak current density of MB at dsDNA was noticeably higher than at ssDNA. These trends were in line with those of Pan et al. (2007) [18], who noted a similar result for a gold electrode. The results seem to indicate that dilution agent (MPA) may cause the difference.

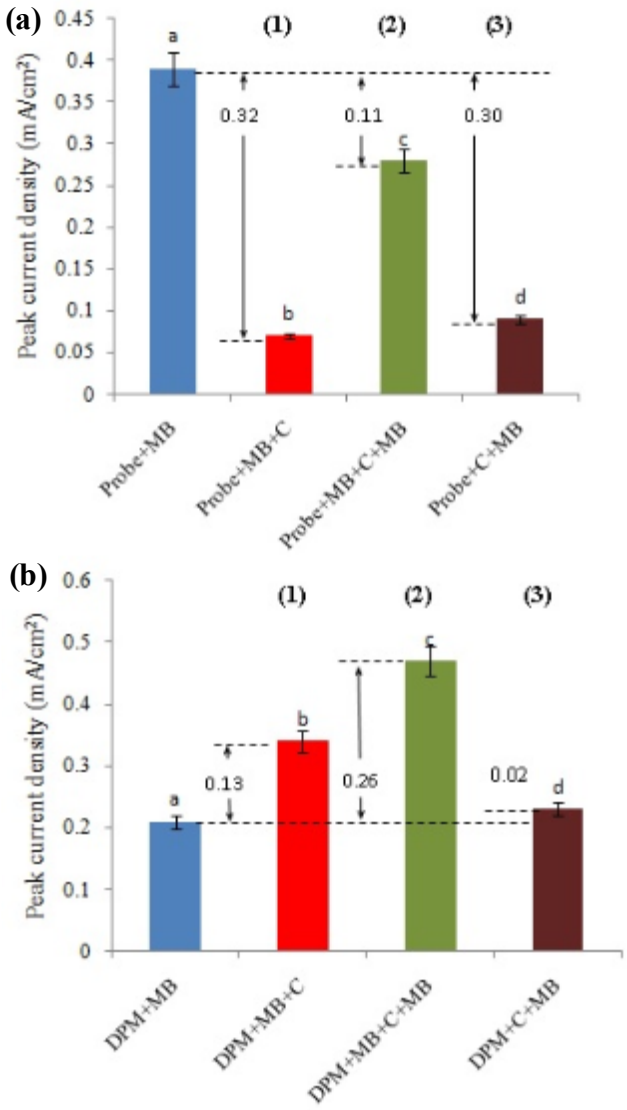

Figure 2. Histogram of SWV signals for (A) thiol-labeled ssDNA probe, (B) thiol-labeled ssDNA probe diluted with MPA(1:5). For three different mechanisms MB accumulation at (a) ssDNA modified CFME, (b) before hybridization, (c) before and after hybridization, and (d) after hybridization. MB accumulation: 5 min in 20 mM Tris-HCl buffer (pH 7.4) containing 20 µM MB. Measurement of accumulated MB in 50 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4). Error bars show the standard deviation of three experiments.

3.2. Dilution agent effect on thiol-labeled DNA biosensor

The overall performance of electrochemical DNA hybridization biosensors is strongly dependent upon the surface chemistry used for interfacing the DNA probe and the electrode transducer.

All of the graphics according to mechanism of MB binding are considered when undiluted thiol-labeled ssDNA (probe) are used, the signal of MB was high in comparison with the one obtained from dsDNA modified electrode (Fig. 2). However, when diluted thiol-labeled ssDNA solution was used, the signal of MB was low in comparison with the one obtained from dsDNA-modified electrode. In the report of Ozsoz, DNA lay down on electrode surfaces (Fig. 3a,b). When ssDNA was assembled on CFME as Fig. 3a, a high MB reduction signal would be observed, because MB had a strong affinity for the guanine and could interact most guanines easily. But after the formation of DNA duplex (Fig. 3b), the guanines were wrapped in the duplex structure, thus preventing MB-guanines interactions. In contrast, when ssDNA was assembled on CFME in a vertical approach via dilution of a probe as Fig. 3c, the MB could interact with the guanines easily even after the formation of DNA duplex (Fig. 3d). Because the DNA duplex can be used as an electron transfer pathway [26,27], the signal of MB reduction can be transferred to the electrode. Therefore, DNA hybridization did not lead to the decrease of MB redox currents in this configuration.

In order to obtain this distribution on the surface, thiol-labeled ssDNA probe diluted with MPA which provides to covering the electrode surface completely and also enables enough space for

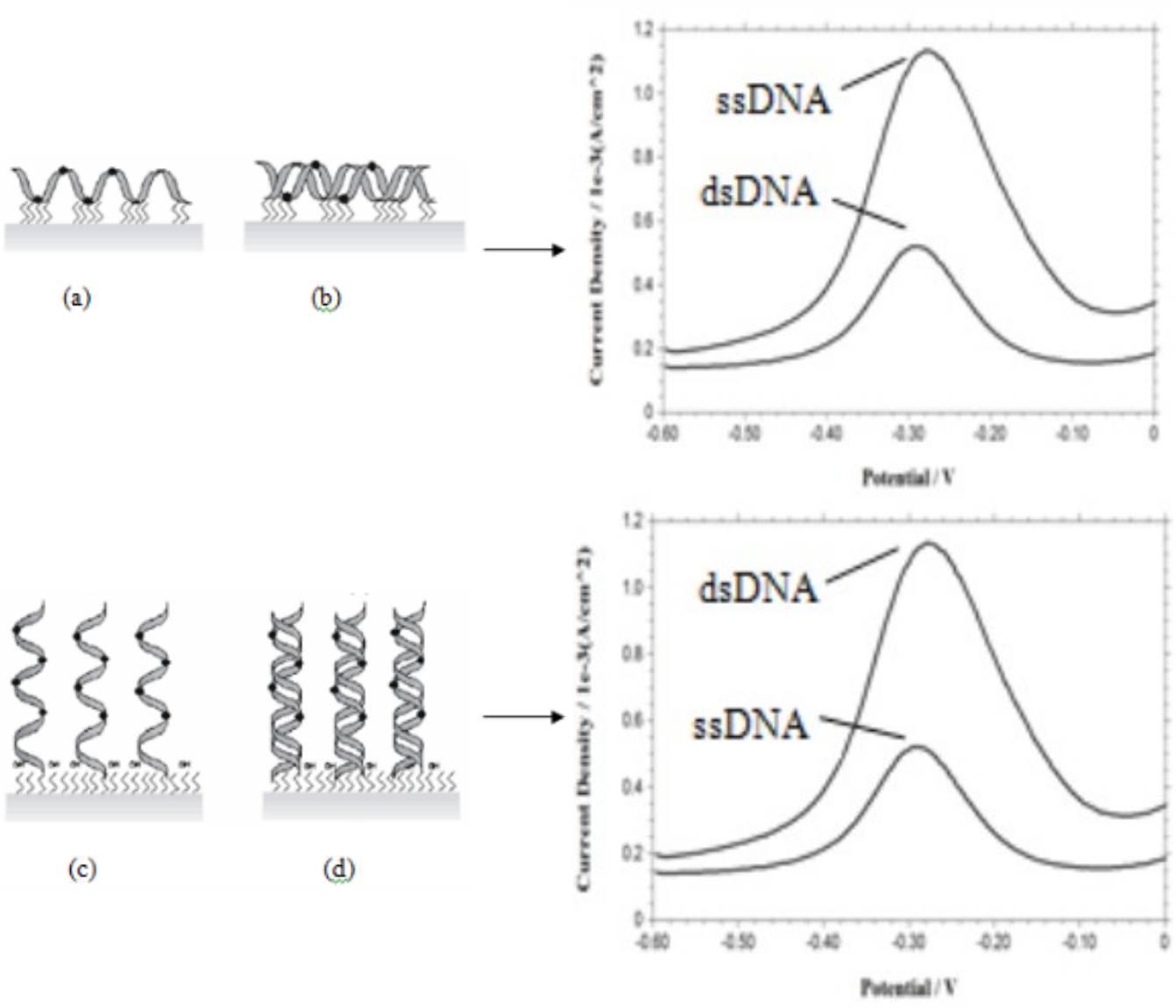

Figure 3. In a) ssDNA and b) dsDNA lies down on electrodes for thiol-labeled ssDNA probe while in c) ssDNA and d) dsDNA are assembled on electrode for thiol-labeled ssDNA probe with diluted MPA such that they are in a vertical configuration. (represents guanine) Pan et al. (2007).

hybridization reaction. Before exposure to MPA, the HS-ssDNA molecules interact with AuNPs-deposited CFME surface trough both the nitrogen-containing nucleotide and the sulphur atom of the thiol group. After exposure to MPA, the thiol groups of MPA compete with the nucleotide bases to interact with the electrode surface [28] Fig. 3c,d.

3.3. Selectivity of the DNA biosensors

In this study, the selectivity of these DNA biosensors was also evaluated using noncomplementary and complementary DNA base sequences. Table 1 shows the peak current densities for probe DNA hybridized with its noncomplementary and complementary base sequence. The peak current density differences (ÄIp=Ip_{ssDNA} - Ip_{dsD-}

Table 1. The peak current density difference (ÄIp) for different DNA sequences

Types of probe		Probe sequence	Noncomplementary sequence (NC)	Complementary sequence (C)
Probe [a]	Ip (mA/cm^2)	0.39	0.34	0.07
Probe [a]	ΔIp[*](mA/cm^2)	0.39	0.05	0.32
DPM [b]	Ip (mA/cm^2)	0.21	0.25	0.47
DPM [b]	ΔIp[*](mA/cm^2)	0.21	0.04	0.26

[*] ΔIp=Ip_{ssDNA} - Ip_{dsDNA}, [a] Thiol-labeled ssDNA probe (18-base sequence)
[b] Diluted thiol-labeled ssDNA probe with 3-mercaptopropionic acid (18-base sequence)

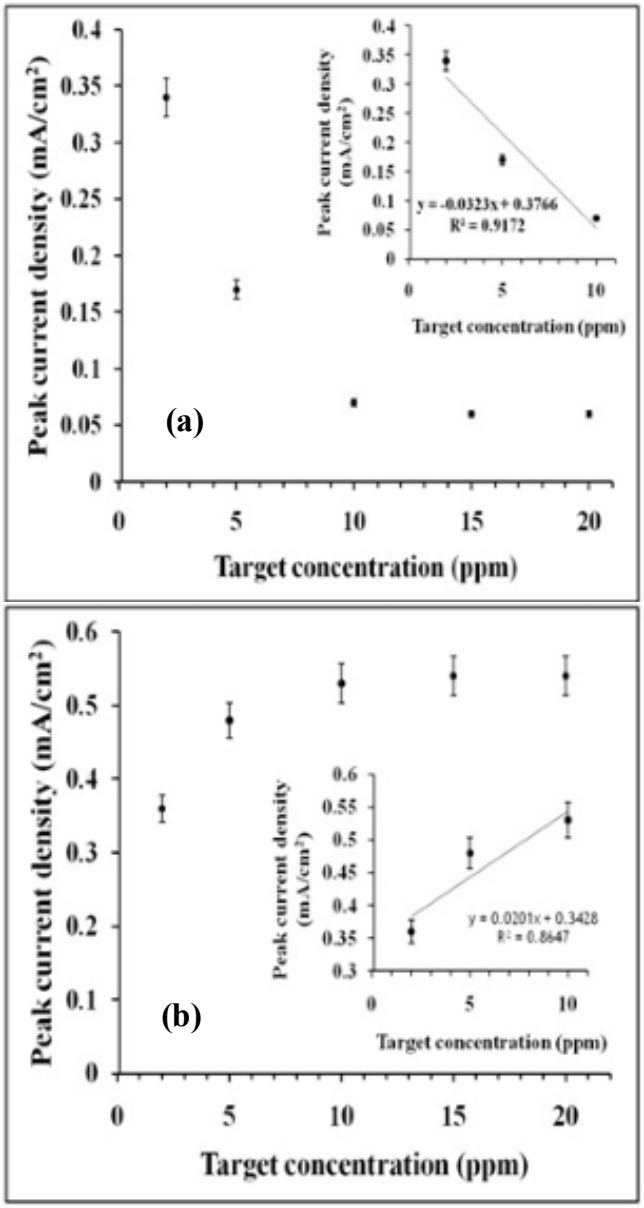

Figure 4. Calibration plots of MB peak current density against DNA target concentration for (A) thiol-labeled ssDNA probe, (B) thiol-labeled ssDNA probe diluted with MPA(1:5). MB accumulation: 5 min in 20 mM Tris-HCl buffer (pH 7.4) containing 20 μM MB. Measurement of accumulated MB in 50 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4). Error bars show the standard deviation of three experiments.

$_{NA}$) were 0.05 mA/cm^2 and 0.32 mA/cm^2 for probe, 0.04 mA/cm^2 and 0.26 mA/cm^2 for DPM, respectively. Because hybridization did not happen effectively due to the sequence mismatch between the modified ssDNA and the noncomplementary base sequence there was not considerable current density difference change for the ssDNA modified CFME and its hybridization with the noncomplementary base sequence. This implies that the surface characteristics of the ssDNA modified CFME was not altered after its interaction with the noncomplementary base sequence. On the other hand, when the ssDNA modified CFME interacted with the complementary target sequence in solution, the peak current density for the MB reduction decreased remarkably. This decrease in current density obviously demonstrated that the ssDNA modified on the CFME effectively hybridized with its complementary target sequence, reducing the intercalation level of MB on the modified CFME because of the steric inhibition effect of MB packing. These results show that the fabricated DNA biosensor can distinguish noncomplementary and complementary target DNA.

3.4. Analytical performance of the each biosensor

Under the optimal conditions, the analytical performance of the fabricated DNA biosensors was investigated using the probe DNA to hybridize with the different concentrations of DNA sequences. Fig. 4 shows the SWVs of the probe modified electrode at various complementary target DNA concentrations. The optimum DNA target concentration was determined as 10 ppm for undiluted probe in Fig. 4A since the minimum MB signal was seen while the probe-modified CFME was subjected to 10 ppm target-including the solution. Therefore, it was thought that complete coverage of the electrode surface with the hybrid was formed with the complementary target. The best DNA target level was 10 ppm for DPM in Fig. 4B because the maximum MB signal was determined once the probe-modified CFME was subjected to 10 ppm target including diluted solution.

The calibration curve showed that the peak current values decreased as the concentrations of the complementary target DNA increased, and it presented good linearity with the concentration of the complementary target DNA from 2 to 10 ppm, with a regression equation of Ip(mA/cm^2) = -0.0323C(ppm) + 0.3766, R^2 = 0.9172. The detection limit for the target DNA was determined as 6.50 ppm from S/N = 3 for the thiol-labeled probe in Fig. 4A. The peak current increased as the concentrations of the complementary target DNA increased, and it was linear with the concentration of the complementary target DNA from 2 to 10 ppm, with a regression equation of Ip(mA/ cm^2) = 0.0201C(ppm) + 0.3428, R^2 = 0.8647. The detection limit for the target DNA was calculated as 8.56 ppm from S/N = 3 for DPM in Fig. 4B. A comparison between biosensors used in this study and previously reported DNA biosensors based on different electrode types using MB as hybridization indicator was shown in Table 2. Although the results for biosensor performance seems to be comparable to the performance of the reported results by Wang et al. [29], in the recent researches some glassy carbon, screen printed carbon paste electrode configurations showed very high sensitivity, low detection limit and linear range [30,31]. The objective of the study was to investigate the potential performance and the design possibilities of a CFME based DNA biosensor by using MB as a hybridization indicator. The performance of the biosensor representing by the biosensor parameters which are regression coefficient, linear range and detection limit clearly indicated that CFME was capable of using as a mediated electrode for DNA biosensor. From this respect, the obtained experimental parameters can be developed through the further investigations, and the performance of CFME based DNA biosensors can be increased.

4. CONCLUSION

We have presented a novel approach for monitoring interactions between methylene blue and DNA using carbon fiber based DNA biosensor. Three different hybridization mechanism were developed between MB and DNA to obtain more specific and selective results. The consistency of the results was tested with the differently designed probes. These results demonstrated that difference of the MB signal in first proposed mechanism (Probe+MB+C) was the most discriminative for thiol-labeled DNA probe. In the contrary, when diluted thiol-labeled ssDNA probe was used, the second proposed mechanism (DPM+MB+C+MB) should be preferred because of signal difference. This study shows that before or after hybridization, MB accumulation is related to probe configuration. However, thiol-labeled ssDNA probe with together dilution agent is more important to solve the MB binding problem. In this study, carbon fiber microelectrodes wereas used to reveal opportunities for material science.

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Table 2. Comparison between proposed electrochemical DNA biosensors based on CFMEs and other previously reported electrochemical DNA biosensors based on gold electrodes or modified electrodes with gold nanoparticles using MB as hybridization indicator

DNA biosensor method	Regression coefficient, R^2	Linear range, M	Detection limit, M	Ref.
Thiolated DNA immobilized gold electrode		2.0×10^{-8} to 2.0×10^{-6}	1.0×10^{-8}	[29]
Based on glassy carbon electrode modified with gold nanoparticles and graphene	0.997	1.0×10^{-12} to 1.0×10^{-7}	2.0×10^{-13}	[30]
Based on glassy carbon electrode modified with gold nanoparticles and decorated reduced graphene oxide	0.996	1.0×10^{-15} to 1.0×10^{-6}	35×10^{-18}	[31]
Probe [a] +MB+cDNA*	0.917	3.6×10^{-7} to 1.8×10^{-6}	1.18×10^{-6}	This work
DPM [b] +MB+cDNA*+MB	0.865	3.6×10^{-7} to 1.8×10^{-6}	1.55×10^{-6}	This work

[a] Thiol-labeled ssDNA probe (18-base sequence), [b] Diluted thiol-labeled ssDNA probe with 3-mercaptopropionic acid (18-base sequence), * Complementary sequence