

Modeling of DNA Hybridization Detection Using Methylene Blue as an Electroactive Label

B. Sehatnia^{a,b}, F. Golabi^c, M.H. Pournaghi-Azar^d, R.E. Sabzi^a and M.S. Hejazi^{c,e,*}

^aDepartment of Chemistry, Faculty of Science, Urmia University, Urmia, Iran

^bArtemia & Aquatic Animals Research Institute, Urmia University, Urmia, Iran

^cFaculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

^dElectroanalytical Chemistry Laboratory, Faculty of Chemistry, University of Tabriz, Tabriz, Iran

^eDrug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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Mathematical modeling of methylene blue (MB) signal in ssDNA and dsDNA on pencil graphite electrode is described. A DNA biosensor was developed based on MB signal. The probe and target DNAs were 20 mer oligonucleotides corresponding to consensus sequence of HPV major capsid protein L1 gene. Hybrids of various complementary and non-complementary oligonucleotides with the probe were considered as dsDNA with different hybridization degrees. Modeling was developed by incorporation of only the stable forms of dsDNA hybrids. Effect of hybridization degree on current signal in various forms was studied. A factor named AHP (Average Hybridization Percentage) for verifying the hybridization events was defined. Results showed that there is a significant mathematical relation between the calculated AHP and MB signals.

Keywords: Biosensor, Pencil graphite electrode, Methylene blue, Modeling, DNA hybridization

INTRODUCTION

Electrochemical biosensing of DNA targets is rapidly developing and is on great demand due to its high sensitivity, small dimensions, low-cost/low-volume, and compatibility with microfabrication technology [1]. Electrochemical biosensors' function is based on DNA hybridization for which many strategies including immobilization of a probe on an electrode for tracking DNA hybridization events have been developed [2]. Electroactivity of single stranded and double stranded DNA chains relies on the electroactivity of DNA bases composing the chains [3]. Because of the wide potential window of carbon electrodes, various groups have used

alternative forms of carbon electrode such as carbon paste [4], pencil lead [5-7] and screen printed electrodes [8] for DNA biosensor development.

Methylene Blue (MB) has been widely used as an electrochemical electroactive label for monitoring DNA hybridization reaction due to its various affinities to ssDNA and dsDNA [9-14]. MB specifically binds to the guanine bases in ssDNA [15] and a high signal is observed. However, following hybridization of probe with complementary DNA, a lower signal current is observed due to less amount of MB that could incorporate into dsDNA. This is due to less or no accessibility of the guanine bases in dsDNA. We used MB as an electrochemical indicator to monitor human interleukin-2 (IL-2) DNA hybridization reaction [16]. We also developed an electrochemical DNA biosensor using sense strand of HPV

*Corresponding author. E-mail: saeidhejazi@tbzmed.ac.ir

Probe immobilization on the PGE. Immobilization of the probe was carried out by applying the optimized potential of -0.5 V for 300 s to the activated PGE immersed in 0.5 M acetate buffer solution (pH = 4.80) containing 20 mM NaCl and 10 ppm probe [17]. Then the electrode was rinsed with sterilized and deionized water for 10 s.

Hybridization. Following the immobilization of the probe on the activated PGE, the PGE was inverted upside down. About 10 μ l of the mixture DNA solution containing 10 ppm target DNA with the same concentration of non-complementary oligonucleotides was pipetted directly onto the electrode surface. The hybridization was allowed to proceed for 2 min at room temperature. Then the accumulation of MB on the PGE and voltammetric determination were carried out. In order to have the maximum interaction of the probe with DNAs, the DNAs were denatured before applying the mixed solutions of the DNAs. To denature DNA, the solution was placed in boiling water for 2 min and then immediately cooled in ice. This kind of denaturation treatment breaks down double stranded DNA bonds. In denatured forms, the chains will have minimum level of hybridization and thus maximum number of non-hybridized bases will be available for hybridization with the probe.

MB accumulation on PGE. MB was accumulated on the probe-modified PGE by immersing it into 20 mM Tris-HCl solution (pH = 7) containing 25 μ M MB and 20 mM NaCl for 5 min with 200 rpm stirring without applying any potential. The PGE was rinsed with Tris-HCl buffer solution. This protocol was repeated prior to every voltammetric determination.

The same protocol was applied for MB accumulation on the bare-electrode and the subsequent hybridization.

Electrochemical measurement. Square wave voltammetry (SWV) was used to detect the reduction signal of MB. Voltammetric determinations were carried out in 0.5 M Tris-HCl buffer containing 20 mM NaCl and sweeping the electrode potential between -0.8 - +0.1 V using a step potential of 50 mV. Reproducibility of response current of the HPV DNA on PG electrode was investigated at least three times at 0.5 M acetate buffer (pH = 4.8).

Theory and structure. Our results showed that MB reduction signal after probe hybridization with complementary DNA is much less than signals related to the non-

complementary DNAs. MB signal of the probe modified electrode was 88.7 μ A which dropped to 40.9 μ A following hybridization of the probe with its complementary DNA. MB signal for hybridization of five non-complementary DNAs varied between 65.3 μ A for 18sr DNA and 74.6 μ A for Lb16s, respectively. Thus, MB signal for the non-complementary DNAs hybridized with the probe is lower than the probe signal and higher than that of complementary-probe hybrid signal. Measurement of MB signal following hybridization of non-complementary DNAs with the probe showed that this signal varies between probe and probe-complementary hybrid signals. Regarding the probe alone, which is considered as a single stranded DNA, the peak current had its highest amount.

The same results were obtained for mixed solutions composed of two or more complementary and/or non-complementary DNAs. When a non-complementary DNA was mixed with the probe's complementary DNA, MB signal was a little higher than the signal of probe-complementary DNA hybrid. This is due mainly to non-specific interactions between non-complementary oligonucleotides with the probe and also with the complementary oligonucleotide leading to inaccessibility of some probe molecules to its complementary DNA. Consequently, a portion of probe molecules are incorporated in hybridization with the complementary DNA. With respect to two non-complementary DNA mixtures with 100% complementary to each other; *i.e.* hIL-2 and cIL-2; MB signal was lower than that of probe alone but very close to it. This is perhaps due to the fact that nearly all molecules of the two complementary DNAs are hybridized together and only a very small portion of these molecules interact with the probe and target DNA and consequently the MB signal drops down and gets very close to the probe signal.

In this study, a formula for the relationship between MB signal and DNA hybridization events is suggested. In these predictions, DNA self-hybridization and inter-DNA molecules hybridization are excluded.

Modeling Theory

The findings show that MB signal is related to DNA hybridization events properly by a mathematical behavior. To describe the hybridization events of the non-complementary DNAs and have a quantitative approach to them, we evaluated all the possible hybridization forms between the probe and the

non-complementary DNAs according to hybridization melting temperature (T_m). Considering that all DNAs were used with the same concentration, its effect was omitted. The T_m for hybridization is calculated as:

$$T_m = 2 \times (\text{number of A-T connections}) + 4 \times (\text{number of G-C connections}) \quad (1)$$

Because of low stability and dissociation of dsDNA hybrids with lower T_m , we considered 15 °C and 20 °C as two thresholds. Therefore, the hybrids with T_m lower than the thresholds were excluded and only the hybrids with T_m s higher than the thresholds were included in the calculations.

It is well known that higher T_m for a particular hybridization form means more hydrogen bonds between two chains of the hybrid and higher and stronger hybridization energy. Accordingly, the incidence possibility of hybrids with higher T_m s will be more than the ones with lower T_m s. Considering that two DNA chains make various hybridization forms and in order to incorporate all hybrid forms, we considered each form of hybridization as a part of the total hybridization according to its T_m . Based on this frame, a factor as average hybridization percentage (AHP) was defined for each hybridization form. AHP is calculated as:

$$AHP = 100 \times \sum (K_i \times P_i) \quad i = 1, \dots, n \quad (2)$$

In this formula, n is the number of possible hybridization forms of each DNA with T_m equal to or above the considered threshold, P_i is the ratio between the number of hybridized nucleotides of the probe to total number of probe nucleotides and K_i is the normalized coefficient of incidence possibility of each hybridization form which is calculated as:

$$K_i = T_{m_i} / \sum T_{m_j} \quad j = 1, \dots, n \quad (3)$$

For instance, included hybrid form of hIL-2 with the probe and their calculated T_m s are as follows:

HPVp: 5'-GTA TCT ACC ACA GTA ACA AA -3'
 hIL-2: 5'-GGA GGA AGT GCT AAA TTT AG -3'

Form 1:



$$P_1 = 5/20$$

$$T_{m_1} = 16 \text{ }^\circ\text{C}$$

Form 2:



$$P_2 = 5/20$$

$$T_{m_2} = 18 \text{ }^\circ\text{C}$$

Form 3:



$$P_3 = 8/20$$

$$T_{m_3} = 22 \text{ }^\circ\text{C}$$

Form 4:



$$P_4 = 7/20$$

$$T_{m_4} = 18 \text{ }^\circ\text{C}$$

Thus, using the above-listed equations, AHP related to hIL-2 DNA is 32% for T_m equal to or over 15 °C and 40% for T_m equal to or over 20 °C.

RESULTS AND DISCUSSION

Primary Investigation

We have already reported the optimum conditions for HPV DNA hybridization and MB signal is presented in Table 1 [17]. Following the immobilization of the probe on the PGE (ssDNA) and hybridization of target DNA with the probe (dsDNA), the current peak for the reduction of MB accumulated on the electrode was measured in both cases. Results showed that MB signal increased to 88.7 μA in ssDNA compared to bare PGE signal which was 39.5 μA as

Table 1. Obtained Peak Currents for MB and AHPs at Two TMs for Data of Ref. [17]

DNA	MB Signal Peak current (μA)	AHP (%) $T_m \geq 15^\circ\text{C}$	AHP (%) $T_m \geq 20^\circ\text{C}$
Bare	39.5	-	-
Com.	40.9	100.0	100.0
18sr	65.3	37.0	40.0
hIL-2	68.5	32.0	40.0
HCV	72.2	31.0	45.0
Lb16s	74.6	35.0	38.0
chIL-2	71.9	35.0	40.0
Probe	88.7	0.0	0.0

Table 2. Obtained Peak Currents for MB and AHPs at Two TMs

DNA	MB Signal Peak current (μA)	AHP (%) $T_m \geq 15^\circ\text{C}$	AHP (%) $T_m \geq 20^\circ\text{C}$
Bare	39.5	-	-
Com.	40.9	100.0	100.0
Com+18sr	45.8	59.8	82.5
Com+hIL-2	52.7	60.0	82.2
Com+chIL-2	47.9	73.6	83.3
Com+HCV	47.6	55.7	83.0
Com+Lb16s	48.1	61.7	72.0
Com+hIL-2 +chIL-2	49.8	40.0	58.0
hIL-2 +chIL-2	85.8	11.7	6.0
Probe	88.7	0.0	0.0

shown in Fig. 1. The current signal remarkably decreased from 88.7 μA to 40.9 μA following the interaction of probe with its complementary DNA. These results confirmed the sensitivity of the proposed electrode in detecting its target DNA.

MB Signal Value Following Interaction of the Probe with Various Oligonucleotides

As shown in Table 2, a significant decrease in MB signal was observed following hybridization of complementary oligonucleotide with the probe. This may be attributed to less MB accumulation on the dsDNA caused by the inaccessibility of MB to the guanine bases [11,12] or may be due to a steric inhibition of the reducible groups of MB packed between the bulky double helix of the DNA hybrids [9]. One may conclude

that the decrease in the MB signal represents the extent of the hybridization at the electrode surface. The presence of chIL-2 or hIL-2 in the mixed solutions has had less effect on the hybridization event between probe and HPVc target. However, a slight increase was observed for MB signal from 40.9 μA to 47.9 and 52.7 μA for chIL-2 and hIL-2, respectively. This small increase may be attributed to the partial hybridization occurring between HPVc and non-complementary oligonucleotide and also between probe and non-complementary oligonucleotides in the mixture solution. The SW voltammograms of ternary mixture of HPVc, chIL-2 and hIL-2 and also binary mixture of chIL-2 and hIL-2 displayed a significant decrease in MB signal to 49.8 μA and 85.8 μA , respectively. One can conclude that the interaction of HPVc

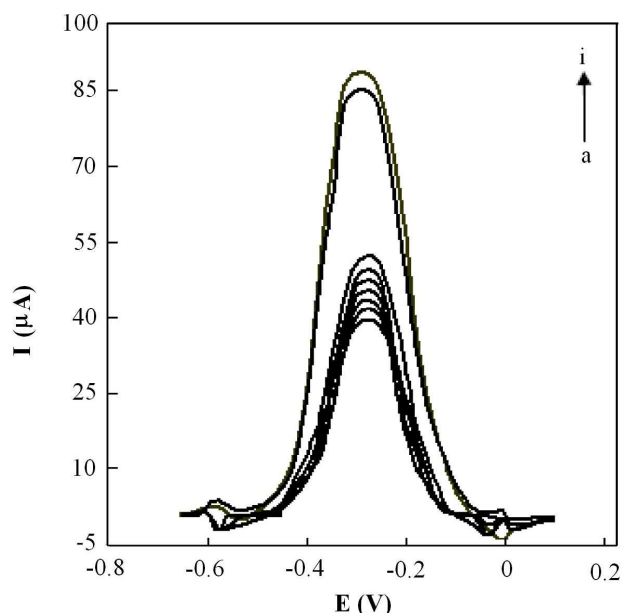


Fig. 1. The Square wave voltammograms of accumulated MB at bare PGE (a), after hybridization treatment of probe modified electrode with solution containing: only HPVc oligonucleotide (b), binary mixture of HPVc and 18sr (c), binary mixture of HPVc and chIL-2 (d), binary mixture of HPVc and Lb16s (e), ternary mixture of HPVc, chIL-2 and hIL-2 (f), binary mixture of HPVc and hIL-2 (g), binary mixture of chIL-2 and hIL-2 oligonucleotides (h) and accumulated MB at probe-modified PGE (i).

with chIL-2 and hIL-2 in their ternary mixture has less effect on the hybridization event between probe and HPVc target compared to chIL-2 and hIL-2 binary mixtures. This is due to the complete hybridization between chIL-2 and hIL-2 oligonucleotides that leads to high degree of hybridization between these oligonucleotides. The dsDNA chains cannot hybridize completely neither with probe nor with HPVc and consequently cannot interfere with probe-DNA hybridization.

Binary mixture of chIL-2 and hIL-2 oligonucleotides in the absence of the HPVc oligonucleotide did not lead to significant MB signal decrease and the signal was nearly equal to that of the probe signal. In the binary mixture of HPVc and 18sr a remarkable decrease to 45.8 μA in the MB signal was observed. The SW voltammograms of binary mixture of

Lb16s and HPVc demonstrated a significant decrease to 48.1 μA in the MB signal.

The SW voltammograms for the accumulated MB in the solution containing HPVc and HCV was 47.6 μA . These data clearly confirmed the selectivity of the developed electrode in detecting and discriminating the target DNA from non-complementary DNAs in mixed solutions.

Mathematical Modeling

We have employed MB as the label for biosensing of target DNA corresponding to human papilloma virus major capsid protein L1 gene. Our findings showed that MB signal after probe hybridization with complementary DNA was much less than signals related to the non-complementary DNAs. Our data indicate that MB signal of probe modified electrode was 88.7 μA which dropped to 40.9 μA following hybridization of the probe with its complementary DNA. MB signal for hybridization of five non-complementary DNAs varied between 63.3 μA for 18sr DNA and 74.6 μA for Lb16s, respectively. MB signal for the non-complementary DNAs hybridized with probe is lower than the probe signal and higher than that of complementary-probe hybrid signal. Measurement of MB signal following hybridization of non-complementary DNAs with probe showed that this signal varies between probe and probe-complementary hybrid signals. Regarding probe alone, which is considered as a single stranded DNA, the peak current had its highest amount.

When combined, the same results were obtained for two or more complementary and/or non-complementary DNAs. When a non-complementary DNA is mixed with the probe's complementary DNA, MB signal is a little higher than the signal of probe-complementary DNA hybrid. This is because of inaccessibility of some probe nucleotides for hybridization with its complementary nucleotides due to steric inhibition of the probe by non-complementary DNA chains. Consequently, a portion of probe molecules are incorporated in hybridization with the complementary DNA. This results in decreasing the overall hybridization percentage to be lower compared to that of complementary DNA alone and eventually the measured MB signal will become higher. With respect to two non-complementary DNA mixtures with 100% complementary; *i.e.* hIL-2 and chIL-2; MB signal was lower than that of probe alone but very close to it. This is presumably due to the fact

that, nearly all molecules of the two complementary DNAs are hybridized together and only a very small portion of these molecules take part in hybridization with the probe and target DNA and consequently the MB signal drops to very close to the probe signal.

In this study, we aimed to discriminate HPV in mixed DNA samples and formulate the relationship between MB signal and DNA hybridization events. In these predictions, as mentioned before, DNA self-hybridization and inter-DNA molecules hybridization were excluded.

First, we considered every possible hybridization forms for all oligonucleotides in pure solutions [17]. Then, AHP for each DNA for the two categories, $T_m \geq 15^\circ\text{C}$ and $T_m \geq 20^\circ\text{C}$ was calculated. The results of our calculations are shown in Table 1.

Then, we studied data related to the mixed solutions of complementary and/or non-complementary DNAs. In this case, hybridization forms of different DNAs are incorporated according to the above-mentioned equations. Again, for each case we considered two categories, $T_m \geq 15^\circ\text{C}$ and $T_m \geq 20^\circ\text{C}$, and calculated AHP for each category (Table 2). When the solution is a mixture of only hIL-2 and chIL-2 DNAs, we see that the MB signal is near the signal related to the use of probe alone. That is because most of hIL-2 and chIL-2 are hybridized together and an average of less than one sixth of these DNAs may hybridize with the probe. The reason for this is that hybridization of hIL-2 and chIL-2 is 20 out of 20 matches with T_m equal to 54, but the hybridization of hIL-2 and chIL-2 to the probe is at the most 8 out of 20 matches for both with T_m s of 22 and 20, respectively.

In case of using probe's complementary DNA mixed with other non-complementary DNAs, the MB signal is higher than the case with only complementary DNA but still lower than probe alone.

The diagrams of MB signals related to the mixed solutions of DNAs versus their calculated average hybridization percentage (AHP) for $T_m \geq 15^\circ\text{C}$ and $T_m \geq 20^\circ\text{C}$ are shown in Figs. 2 and 3, respectively. It seems that there is a near-linear relationship between the two variables that confirms our findings.

According to Fig. 2, when considering all cases with $T_m \geq$

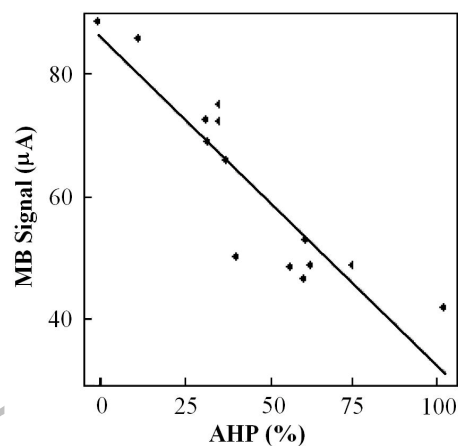


Fig. 2. Plot of the MB signal peak currents vs. average hybridization percentage for $T_m \geq 15^\circ\text{C}$. ($R^2 = 0.82$).

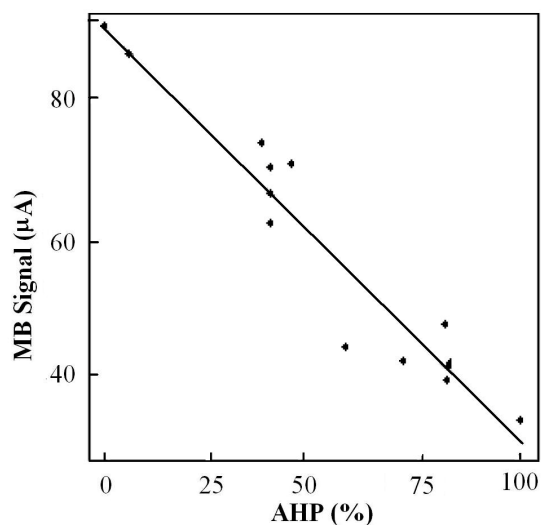


Fig. 3. Plot of the MB signal peak currents vs. average hybridization percentage for $T_m \geq 20^\circ\text{C}$. ($R^2 = 0.93$).

15°C , R-square is 0.82 and so R equals to 0.91. Furthermore, Fig. 3 shows R-square of 0.93 for $T_m \geq 20^\circ\text{C}$ and so R equals to 0.96 in this case. Thus, because of lower thermal stability of hybridization forms with T_m under 20, and also lower R

incorporated in these cases, we conclude that our modeling will yield its best results when stronger and more possible forms of hybridization with $T_m \geq 20$ °C are considered.

CONCLUSIONS

A biosensor for the detection and discrimination of HPV DNA in a mixture of different DNAs was developed. The sensitivity and selectivity of the electrode was approved by detecting the HPV complementary in different mixtures of non-complementary DNAs. The decrease of the reduction signal of MB after DNA hybridization reflects the extent of the DNA hybrid formation. Our results showed that MB signal in DNA biosensors follows a logical manner and a mathematical relation between hybridization events and MB signal is suggested. To model this behavior, we defined AHP factor as average hybridization percentage and calculated it for each hybrid. Results indicate that there is a mathematical relation between the calculated AHP and MB signal especially when stable forms of hybridization are considered. Further studies are suggested to investigate other factors which may have an effect on MB signal.

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