Direct measurement of the melting temperature of supported DNA by electrochemical method

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Received July 18, 2003; Revised September 29, 2003; Accepted October 7, 2003

ABSTRACT

The development of biosensors based on DNA hybridization requires a more precise knowledge of the thermodynamics of the hybridization at a solid interface. In particular, the selectivity of hybridization can be affected by a lot of parameters such as the single-strand (ss)DNA density, the pH, the ionic strength or the temperature. The melting temperature, $T_m$, is in part a function of the ionic strength and of the temperature and therefore provides a useful variable in the control of the selectivity and sensitivity of a DNA chip. The electrochemical technique has been used to determine the $T_m$ values when the probe is tethered by a DNA self-assembled monolayer (SAM). We have built a special thin layer cell, which allows the recording of the cyclic voltammogram under controlled temperature conditions. $T_m$ has been determined by recording the thermogram (current versus temperature) of a redox indicator on a double-stranded hybrid (dsDNA) modified electrode and comparison with the corresponding ssDNA response. $T_m$ of supported DNA varies linearly with the ionic strength. The stability of the SAMs has been considered and comparison between $T_m$ in solution and on a solid support has been discussed.

INTRODUCTION

The knowledge of the melting temperature, $T_m$, is of technological interest to understand the hybridization mechanism involved in a DNA chip. $T_m$ is generally used as a parameter to adjust the properties of the cell in a DNA chip. The number of bases of DNA is modified so that $T_m$ is maintained constant for each cell. The $T_m$ value used is that measured in liquid medium. $T_m$ in solution is well determined by spectroscopy and many empirical formulas were developed to account for both the ionic strength and the mole fraction of G-C base pairs (1,2). More precise $T_m$ values can be obtained through on-line $T_m$ calculators (http://www.idtdna.com/) (3).

However, the environment of oligomers can differ between a solid interface and the bulk solution due to difference between ionic strengths. The change in surface charge density induced by the hybridization is expected to influence the $T_m$ value. There is rather scarce information on $T_m$ when DNA strands are immobilized on to a surface.

The difficulty is to measure the true surface temperature of the substrate and therefore find the more appropriate method to heat the DNA support. The nature of the substrate onto which the DNA is immobilized is generally governed by the choice of the signal transduction. For example, in classical tests involving fluorescent or radiolabeled arrays, DNA probes are attached mostly to silica or glass surfaces (4–7) whereas piezoelectric, surface plasmon resonance or electrochemical detection involve nucleic acids immobilized on gold electrodes (4,8,9).

For DNA on silica, the ‘supported’ $T_m$ values have been determined by using fluorescence (10) and impedance measurements (11). The thermodynamic stability of immobilized double-stranded (ds)DNA is different from that of dsDNA in bulk solution and depends on the DNA density, namely the nearest-neighbor interactions (10).

Concerning the DNA attached to gold via an alkanethiol anchor, $T_m$ has been determined by using two-color surface plasmon resonance spectroscopy (12). As the substrate is deposited on a prism, the immobilized DNA is heated indirectly by means of the substrate.

These three techniques give $T_m$ values close to those in solution, varying by $<10^°$C. Nevertheless, some key issues are still unclear: the thermal stability of SAMs is of crucial importance in the determination of $T_m$; aliphatic alkanethiols have been reported to thermally desorb (13–16). The desorption temperature ranges from 37°C to >100°C depending on the aliphatic chain length and on the surface state.

We propose a new approach that can provide an alternative and easy way to both measure the $T_m$ of supported DNA film and verify the stability of the immobilization. The electrochemistry has the advantage of combining the analytical power of electrochemical methods with the specificity of the biological recognition process. The present work aims to

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investigate the capability of the electrochemical methods to determine the $T_m$ of supported DNA. We have adapted the electrochemical cell for heating the substrate within 0.1°C accuracy and measuring the electrical signal of a redox indicator. Different redox indicators have been tested to select the best candidates for thermal studies. The high packing density of the DNA monolayer is controlled using permeability experiments.

In such experiments, the stability of self-assembled monolayer (SAM) versus the applied potential is very important. SAMs have been shown to be stable to potentials between approximately +0.8 and ~1.4 V versus saturated calomel electrode (SCE) depending on the type of thiol derivative and on the properties of the metallic surface (17–22). Within these limits, thiol SAMs do retain structural order and high packing density especially in aqueous medium. Therefore, the indicators used in this study are electroactive within this potential window.

Discussion focuses on the comparison between $T_m$ in solution and on the electrochemical probe to correlate the $T_m$ with the ionic strength.

**MATERIALS AND METHODS**

**Materials**

Methylene blue (MB) (Aldrich), potassium ferricyanide (Aldrich) and hexaammineruthenium(III) chloride (Aldrich) were used as received at a concentration of 5 × 10⁻⁵, 10⁻⁴ and 10⁻³ M, respectively.

HPSF® purified 5'-TTT TTT TTT TTT -(CH₂)₆-SH-3' and underivatized complement were purchased from MWG Biotech (Evry, France). Mercaptohexyloligonucleotides were stored frozen to prevent oxidation of the thiol.

**Hybridization**

Mercapto- and underivatized oligonucleotides (0.1 mM) were hybridized in 5 mM phosphate/50 mM NaCl by heating to 90°C for 10 min followed by slow cooling to room temperature. In all experiments, hybridization is performed before the immobilization on to the gold electrode.

**Derivatization of gold electrodes**

Au(III) surfaces were prepared by thermal evaporation at a pressure of 10⁻⁶ Pa (0.1 nm/s) of a thick gold layer (175 nm) on to glass plated with 4 nm of chromium. Freshly evaporated gold electrodes were then modified by incubation in 0.1 mM solutions of derivatized DNA duplexes in 5 mM phosphate/50 mM NaCl (pH 7.4) for 18–48 h at ambient temperature. Modified electrodes were rinsed in phosphate buffer prior to use. The DNA density was previously quantified by ³²P-radiolabeling (23). We obtained a surface coverage of 8 × 10¹¹ strands/cm².

**Electrochemistry**

All the electrochemical experiments were carried out in a deoxygenated buffer made of 5 mM phosphate/x mM NaCl (x = 35, 50, 100, 200) Millipore water (pH 7.4). The electrochemical instrumentation used for these experiments includes an EG&P 283 potentiostat connected to a PC. Data collection and analysis were performed using a Princeton Applied Research Software Model 270. The DNA modified electrodes were used as working electrodes. Potentials were measured relative to the Ag/AgCl/NaClₓₜₜₜ reference electrode and then recalculated with respect to the SCE. The electrochemical techniques used are cyclic voltammetry and square-wave voltammetry. We have shown previously (23) that the electrochemical response in particular the signal-to-noise ratio is greatly improved by using square-wave voltammetry. Square-wave voltammetry is a dynamic method in which a train of pulses is applied at a stationary electrode. A rather large amplitude symmetrical square-wave perturbation is applied, each cycle of the square-wave coinciding with one cycle of an underlying staircase (Fig. 1A). The waveform is characterized by ΔEₛ, the step height of the staircase, Eₛₕₜ, the half-peak-to-peak amplitude of the square-wave and t, the period of the square-wave excitation. The time parameters may be described alternatively by the frequency, $f = \frac{1}{t}$, or the pulse width, $t_p = \frac{t}{2}$. The pulse width is the characteristic time of the experiment. Current is sampled over sampling interval $t_s$. The current sampled on the forward-going or first half-cycle is referred to as the forward current ($i_f$). The reverse current ($i_r$) corresponds to the second half-cycle. Generally the net peak current ($\Delta i = i_f - i_r$) is used because it is greater than $i_r$ since $i_f$ for a reversible system is opposite in sign to $i_r$ near the peak. $W_{1/2}$ is defined as the net peak half-height width. After optimization of the different parameters, the best results have been obtained with $f = 100$ Hz, $\Delta E_S = 1$ mV and $E_{SW} = 50$ mV. All the square-wave voltammograms have been carried out under these conditions.

**Measurement cell**

A special cell was developed to control the temperature of the DNA modified electrode (Fig. 1B), as described previously (23). The main characteristic of the cell is its small internal volume (3 × 1.5 × 9 mm) thereby permitting us to handle only a few microliters of solution using a microsyringe. Inlets and outlets of liquid of 110 µm in diameter are located in the cell base. The tightness of the cell is ensured by pressing all elements using four screws. All solutions passing through the cell are deoxygenated prior to experiments. Due to the small size of the cell, deoxygenation is not necessary during experiments. The electrode support constitutes the upper part of the cell. Located in the lower part, the auxiliary electrode is a platinum disk of 2 mm in diameter and the reference is a thermodynamic Ag/AgCl/NaClₓₜₜₜ electrode, which relies on chloride concentration in the flow stream. The temperature is controlled within 0.1°C accuracy from 20 up to 80°C using a Peltier heating plate in contact with the electrode support. To ensure a good equilibrium in temperature, electrochemical measurements are performed 10 min after the increase in temperature.

**RESULTS AND DISCUSSION**

**Surface control**

The negative anion Fe(CN)$_6^{3-}$ was chosen since it does not bind to the DNA, a poly-anionic molecule, due to repulsive effects (25) and is electro-inactive even at over-potentials as high as +1 V. Fe(CN)$_6^{3-}$ becomes particularly interesting to
estimate the DNA coverage on the electrode. Therefore, when the electrode is covered with a densely built-up monolayer, the cyclic voltammogram presents a lack of signal. Alternatively, when the gold electrode is partially uncovered, Fe(CN)\(_6^{4-}\) is oxidized on the gold surface leading to a characteristic electrochemical response of ferrocyanide at 0.18 V whose intensity is proportional to the amount of desorbed DNA. Cyclic voltammetry of Fe(CN)\(_6^{4-}\) is used then as a routine method to guarantee a good derivatization of the probe.

**Choice of the redox indicator**

Figure 2 shows the square-wave voltammograms of three redox species at a DNA modified electrode: ferrocyanide Fe(CN)\(_6^{4-}\), hexaammineruthenium(III) Ru(NH\(_3\))\(_6^{3+}\) and MB. The second redox indicator, Ru(NH\(_3\))\(_6^{3+}\), is a groove binding agent (26). Its reduction proceeds through the facilitated diffusion of the ruthenium complex along the grooves of the DNA helix (25). Recording the electrochemical response of Ru(NH\(_3\))\(_6^{3+}\) as a function of temperature does not yield measurable differences. This may be due to the binding mode of Ru(NH\(_3\))\(_6^{3+}\), which remains rather far from the stacked bases responsible for the electron transfer.

The best results have been obtained with MB. The intensity of the square-wave voltammogram is 12t-fold higher than that of Ru(NH\(_3\))\(_6^{3+}\). MB behaves as an adsorbed molecule: in cyclic voltammetry, the peak current varies like \(v\) and the shape of the peak corresponds to a strong adsorbed system (27). MB intercalates into the DNA base stack and therefore participates in electron transfer mediated by the stacked bases. This is the reason why MB has been chosen as the electrochemical intercalator in the following. At ambient temperature, the peak intensity is nearly the same whether the DNA is hybridized or not. The DNA duplex helices are tightly packed on the gold surface necessitating that MB binds at sites close to the DNA/solvent interface. In contrast, diffusion of MB into the single strand (ssDNA) monolayer becomes facilitated (28,29). This phenomenon counterbalances the rapid electron transfer observed with hybridized DNA (30,31) and therefore provides approximately the same signal intensity for both modified electrodes. It is worth noting that the electrochemical study of Ozsoz et al. (28,29), which presents a diminution of the peak height after hybridization, involves DNA strands lying on the surface of the electrode, therefore ET through the \(\pi\)-stacks of the double helical DNA does not occur; in dsDNA, the bases are less accessible than in the ssDNA so the signal of dsDNA is reduced. In our case, the DNA strands form a densely packed monolayer upright oriented with respect to the gold electrode, favorable to ET. The monolayer thickness has been estimated by AFM between 4 and 6 nm for a 15mer oligonucleotide. This corresponds to upright or slightly bent strands. The same observations, obtained by ellipsometry measurements, have also been reported by Kelley and Barton (32).

**Desorption phenomena**

Figure 3 shows the variation of the maximum net peak current of MB in square-wave voltammetry as a function of the electrode temperature varying between 20 up to 80°C by steps
of 0.5°C. When the temperature increases, the signal of MB with both ssDNA and dsDNA grows until a temperature of 28.5°C for ssDNA and 32°C for dsDNA, then falls down until a value of ∆i/WT/1/2 = 1.8 μA.mV⁻¹. Such a bell-shaped curve has been observed with alkanethiol SAMs and imputed to dynamic movements of the SAMs (13,33). Molecular dynamic simulations predict that at a low temperature the chains are orientationally ordered. When increasing the temperature, chain-disordering processes occur passing from a crystalline-like to a melted state. Partial desorption and surface migration of thiolates have also been observed. The experiment has been repeated with other 15mer DNA duplexes containing guanine bases. The thermograms present the same aspect, namely a maximum of the curve coupled with a limiting value of the temperature. The maximum intensity 20% higher with a 15mer DNA containing seven guanines indicates a greater affinity of MB to guanine and cytosine bases (34,35). The current decrease does not depend on the DNA sequence reaching the same limiting value at high temperatures (T > 70°C). Let’s find the possible origin explaining such a current decrease. It is well known that in square-wave voltammetry, moderately slow quasi-reversible redox reactions give responses larger than much faster reversible reactions (36–38). It has been shown theoretically (36) that in the quasi-reversible region, the contributions of the forward and reverse currents to the net current are approximately equal near the maximum net peak height, then the net peak splits into two. Experimentally, when the net intensity decreases, a split has never been observed and both contributions of the forward and reverse currents remain of the same order. Moreover, the forward and reverse peaks were found to be symmetrical even at high temperatures. These results suggest that the increase in the reaction rate induced by the temperature increase cannot explain the current fall. Alternatively, some authors have suggested that a thermal desorption of alkanethiols SAMs can occur (14–16). This can be considered as the ultimate stage of the SAMs dynamic movements’ process. The electrochemical response of MB on a bare gold electrode has been recorded as a function of the temperature (Fig. 3). MB presents the characteristics of an adsorbed system; in particular, the cyclic voltammogram has a shape corresponding to an electrochemical reaction occurring on the electrode surface and the current is proportional to the scan rate (27,39,40). The low current value at the initial temperature reveals a smaller amount of MB adsorbed on the bare electrode compared with the DNA modified electrode. From 20 to 35°C the net peak decreases due to the apparition of a pre-peak (39,41) close to the other peak on the forward current. This induces a broadening of the net peak and a decrease of its height. When increasing the temperature, the two peaks merge. The current of MB at the gold electrode increases as expected for an activated process (42) until ∆i/WT/1/2 = 1.8 μA.mV⁻¹ is reached at T = 70°C, which matches with the minimum of the DNA modified electrode signal at high temperature. Moreover, cyclic voltammograms have been carried out with Fe(CN)₆⁴⁻ on a gold electrode tethered with 5'-TTT TTT TTT TTT TTT-(CH₂)₆-SH-3' hybridized to its complementary strand. A study in function of the temperature shows a lack of electrochemical signal until 60°C (Fig. 4A and B). Then, the electrochemical response of Fe(CN)₆⁴⁻ merges progressively with increasing the temperature and reaches nearly the same intensity as that observed on a bare gold electrode at T = 80°C (Fig. 4A). Note that Fe(CN)₆⁴⁻ is not oxidized on a well covered electrode due to repulsion of the DNA. The electrochemical signal at high temperatures reveals a partial destruction of the DNA monolayer and a strip of the gold surface where oxidation of Fe(CN)₆⁴⁻ can occur. The thermal desorption of thiol SAMs has already been reported for aliphatic alkanethiol SAMs (13–16). Let us define by T₁/2 the temperature corresponding to desorption of half of the monolayer. Figure 4C represents the variation of T₁/2 with the logarithm of the salt concentration. The thermal desorption of the SAMs is dramatically emphasized by an increase of the ionic strength. The sensibility of DNA SAMs to temperatures is of great importance in the practical use of such DNA probes. In particular, it has been shown that hybridization of an ssDNA immobilized by a thiol anchor has to be realized under mild temperature conditions and at rather low ionic strength. **Tₘ determination and effect of ionic strength** We have reported in Figure 5B the literature ‘supported’ Tₘ values (solid lines) and compared them to the corresponding Tₘ in solution (dotted lines). For the 25mer DNA (12) a
A decrease of ~10°C between the DNA in solution and the supported DNA has been observed. Different results have been obtained for the dA20:dT20 duplex: the solution T_m values calculated with an empirical formula (http://www.idtdna.com/) (3) are of the same order as those for DNA on silica (10). In that case, the slope dT_m/dlog(c_{NaCl}) is little different. An easy way to determine the T_m is to compare the electrochemical responses given by both an electrode tethered with an ssDNA, i.e. 5'-TTT TTT TTT TTT-(CH_2)_6-SH-3' and a second electrode tethered with the corresponding hybridized strands (dsDNA).

The signal obtained for both ssDNA and dsDNA increases with increasing temperature up to 28.5°C for ssDNA and 32°C for dsDNA (Fig. 3). Contrary to the rise of the current that is regular for the ssDNA, the one obtained with the dsDNA presents a bulge at 24°C (for a salt concentration of c_{NaCl} = 35 mM). A fine procedure has been developed to determine precisely the temperature related to the peak maxima. The fitted curve has been obtained by calculating a high order polynomial fit on the whole experimental curve after elimination of the experimental points that form the bulge. For example, the curve corresponding to dsDNA with a salt concentration of c_{NaCl} = 35 mM (blue circles in Fig. 3) can be fitted by a polynomial equation of order 6 or more (blue solid line in Fig. 3). Figure 5A shows the difference between the experimental measurements and the ‘regular’ fit (blue solid line in Fig. 3). As the temperature approaches T_m, the progressive separation of the two strands of the helical DNA.

Figure 4. (A) Cyclic voltammetry of 10^{-4} M Fe(CN)_6^{4-} in 5 mM phosphate/50 mM NaCl (black dotted line) at a bare gold electrode at T = 80°C and at a gold electrode modified with 5'-TTT TTT TTT TTT-(CH_2)_6-SH-3' hybridized to its complement at different temperatures: (red solid line) T = 21°C; (green solid line) T = 60°C and (blue solid line) T = 80°C. (B) Peak current height of 10^{-4} M Fe(CN)_6^{4-} in 5 mM phosphate/50 mM NaCl as a function of the temperature (same DNA as A). (C) Variation of the half desorption temperature (T_{1/2}) with the logarithm of the ionic strength (log(c_{NaCl})) for the 15mer duplex 5'-TTT TTT TTT TTT-(CH_2)_6-SH-3' hybridized to its complement and tethered on a gold electrode.
facilitates the diffusion of MB into the DNA monolayer, leading to an increase in the electrochemical response. Then, the complement strand is completely dehybridized and the signal falls down. To make sure that this temperature can be attributed to \( T_m \), which is known to vary as a function of ionic strength, the experiment has been repeated at different salt concentrations (35 mM \( \leq C_{NaCl} \leq 200 \) mM) and the electrochemical thermograms (current versus temperature) have been drawn. For ssDNA a regular curve is obtained while for dsDNA a bulge is observed. The same procedure as described previously has been performed and the results presented in Figure 5. When increasing the ionic strength, \( T_m \) is shifted towards higher temperatures, indicating stabilization by greater Na\(^+\) concentrations. To compare our results with those in solution, we have determined the \( T_m \) of the DNA–MB complex in solution by UV spectroscopy. The equivalent oligonucleotide concentration has been calculated as follows: the electrode area is 0.15 \( \times \) 0.9 \( = \) 0.135 cm\(^2\) on which the DNA density is 8 \( \times \) 10\(^{11}\) strands/cm\(^2\). This corresponds to 1.79 \( \times \) 10\(^{-15}\) mol in a cell volume of 4.05 \( \times \) 10\(^{-5}\) l, i.e. a concentration of 4 \( \times \) 10\(^{-9}\) M. The absorbance for \( \lambda = 260 \) nm has been reported as a function of the temperature carefully measured with a thermocouple plunged into the solution. This melting curve has been repeated with different ionic strengths. The straight line obtained is presented in Figure 5B (blue dotted line). The \( T_m \) of the DNA–MB complex in solution is lowered by 10°C with respect to the DNA without MB in solution. MB intercalates between the bases and therefore provides a deformation of the DNA, which induces a lower stability of the DNA and facilitates deshybridization. Completely different results have been observed with major groove binding agents (43,44), which contribute to increase the DNA cohesion: it has been shown that DNA is stabilized by iridium or ruthenium complexes and \( T_m \) increases with nearly 5–10°C (43,44). When the DNA–MB complex is tethered on a support, the stability increases. The ‘supported’ \( T_m \) varies linearly as a function of \( \log(C_{NaCl}) \) with approximately the same slope as that obtained in solution but shifted by \( >12°C \) compared with the solution. The \( T_m \) increase observed for supported DNA can be interpreted as an increase of the salt concentration in the monolayer. Moreover, it appears that high packing density facilitates some destabilization of hybridized immobilized oligonucleotides and therefore diminished the \( T_m \) (45). Figure 5B regroups our results together with the literature results obtained with a 20 base (10) and a 25 base tethered DNA (12). The melting temperature rises as the DNA length increases and the slope \([dT_m/d(\log C_{NaCl})] \) is of the same order of magnitude whatever the DNA sequence, indicating an identical sensitivity of \( T_m \) to the ionic strength. In the study of Peterlinz et al. (12), comparison between \( T_m \) in solution and tethered on the gold electrode shows a decrease of 5°C for immobilized oligonucleotides. These results seem to be under estimated values due to thermal gradients in the SPR apparatus between the modified prism surface and the thermal regulator as noticed by the authors. In our case, the temperature measurement corresponds well to the temperature of the DNA probe because the Peltier element is directly in contact with the electrode.

**CONCLUSIONS**

A more precise knowledge of the hybridization occurring at a solid interface is important in the development of DNA chip. Our work provides a comparison between tethered and untethered DNA. We have shown that the melting temperature, \( T_m \), of immobilized DNA can be obtained via the square-wave voltammetric response of MB as a function of the temperature probe. The supported melting temperatures vary
linearly with the ionic strength in the same way as that observed in solution and with values 12°C higher: as the ionic strength increases, $T_m$ is shifted towards higher temperatures indicating stabilization by greater Na+ concentration. The sensitivity of $T_m$ to salt concentration is of the same order whether the DNA is immobilized or not. In solution, the insertion of MB between the bases of the helical DNA induces a destabilization of the DNA and therefore a decrease of $T_m$ by $>10°C$. The fixation of the DNA–MB complex on a support tends to stabilize the DNA, probably due to interactions in the DNA monolayer.

Thiol SAMs of DNA on gold have to be used with care; in particular hybridization must be carried out under mild temperature and ionic strength conditions. Greater temperatures can induce desorption of the DNA monolayer.

ACKNOWLEDGEMENTS

This work was supported by a grant from the CNRS (Program ‘Puce à ADN’).

REFERENCES


