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Label-free characterisation of oligonucleotide hybridisation using reflectometric interference spectroscopy

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Abstract The potential of a label-free detection method, reflectometric interference spectroscopy (RIfS), for temperature-dependent DNA hybridisation experiments (for example in single nucleotide polymorphism (SNP) analysis) is investigated. Hybridisations of DNA, peptide nucleic acid (PNA), and locked nucleic acid (LNA) to a single stranded DNA were measured for several temperatures, and the melting curves and temperatures were calculated from the changes in optical thickness obtained. These measurements were performed by hybridising surface-immobilised single stranded oligomers with their complementary ssDNA or with ssDNA containing SNPs at different temperatures. DNA was compared to its analogue oligomers PNA and LNA due to their stability against nuclease. A comparison of melting temperatures demonstrated the higher binding affinities of the DNA analogues. Moreover, a continuous melting curve was obtained by first hybridising the functionalised surface with its complementary DNA at room temperature and then heating up in-flow. Measurement of the continuous melting curve was only possible due to the insensitivity of the RIfS method towards temperature changes. This is an advantage over other label-free detection methods, which are based on determining the refractive index.

Keywords DNA · Peptide nucleic acid (PNA) · Locked nucleic acid (LNA) · Reflectometric interference spectroscopy (RIfS) · Melting temperature · Melting curve

Introduction

The expression of genes and possible modifications is of great interest to those who seek to better understand the new information about cells and their functions arising from the decoding of different genomes [1]. The knowledge obtained will be useful in the development of new therapies and the fight against disease. In the systematic search for genome mutations, especially the most common forms, single nucleotide polymorphism (SNP) [2] plays an immense role in our understanding of interindividual diversity and inherited diseases. High-density DNA arrays provide a versatile and important tool in this field [3, 4], but affinity determination of oligomer duplexes and therefore SNP detection is usually done by melting curve analysis.

Melting curves can be detected by a variety of techniques, including UV absorbance or fluorescence. In the field of the fluorescence methods, an increasing number of measuring systems are being placed at our disposal, such as those based on DNA intercalation [5] or fluorescence energy transfer (FRET) [6]. Although these methods are well established, they do possess some disadvantages, like the rather high sample consumption of UV absorbance measurements, or the fairly expensive and time-consuming use of fluorophores. In addition, the fluorescent labels can influence the interaction of DNA.

Label-free methods, like surface plasmon resonance (SPR) [7, 8], ellipsometry or reflectometry [9, 10] avoid these problems. Moreover, one of the main advantages of these techniques is the ability to determine kinetic and thermodynamic constants.

Refractometric methods like SPR and ellipsometry are very sensitive to temperature variations due to the high impact of temperature on the refractive index. Thus a change of temperature during a measurement is not possible with these methods, and even fast changes of temperature between measurements are technically challenging. Therefore, they are not suitable for melting curve analysis.

Dedicated to the memory of Wilhelm Fresenius.

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By virtue of its stability against temperature variations, reflectometric interference spectroscopy (RIfS) is predestined for the fast measurement of DNA melting curves and melting temperatures.

DNA analogues and modified nucleic acids like peptide nucleic acids (PNA) [11, 12] and locked nucleic acids (LNA) [13–15] are very promising tools in the field of DNA arrays, gene therapeutics and biological recognition. These substances show equal or higher binding affinity to single stranded DNA (ssDNA) and are therefore of research interest in SNP analysis.

In this work we used the direct optical and label-free detection method RIfS, which is based on white light interference at thin layers [16, 17]. Melting curves of surface-immobilised oligomers with 15 nucleotides to their complementary 15 mer DNA were calculated from hybridisation measurements at several different temperatures. Melting temperatures of DNA/DNA, PNA/DNA and LNA/DNA were calculated from the melting curves. Moreover, SNP analysis was performed by hybridising ssDNA with one or two mutations on the same surfaces.

It was also possible to achieve a continuous DNA/DNA melting curve, in contrast to the discrete measurements achieved before, by using a temperature gradient during time-resolved RIfS measurement.

Experimental

Materials

DNA and LNA oligomers were purchased from IBA, Göttingen, Germany. The PNA oligomer was kindly supplied by Applied Biosystems, Weiterstadt, Germany. For immobilisation to the transduction surface, FBMG1 (5'-CGC TAC AAC CTA CAT-3') single stranded DNA, PNA and LNA oligomers were used, which contained a functionalisation of a 5'-amino and a C-6 spacer. For hybridisation, complimentary FBMG1 antisense ssDNA (5'-ATG TAG GTT GTA GCG-3'), FBMG2 ssDNA with one SNP (5'-ATG TA A GTT GTA GCG-3') and FBMG3 ssDNA with two SNPs (5'-ATG TA A G TG GTA GCG-3') were used. The diaminopoly(ethylene glycol) (PEG) with a molecular weight of 2,000 Da was purchased from Rapp Polymere, Tübingen, Germany. 3-Glycidyloxypropyltrimethoxysilane (GOPTS) and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Fluka, Neu-Ulm, Germany. Phosphate-buffered saline (PBS) solution (150 mM sodium chloride; 10 mM potassium phosphate) was prepared with Milli-Q water and titrated to pH 7.4 with 2 M NaOH. For regeneration, a solution of 0.25% sodium dodecyl sulphate (SDS) in Milli-Q water titrated to pH 2.5 with HCl was used. RIfS-transducer chips of 1 mm-thick D 263 glass with layers of 10 nm Nb₂O₅ and 330 nm SiO₂ were obtained from Unaxis Balzers AG, Balzers,

Lichtenstein. Common chemicals of analytical grade were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany or Merck KGaA, Darmstadt, Germany.

Methods

RIfS is based on multiple reflections at thin layers. To this end, white light is guided with an optical fibre to the transduction layer. At each interface between different refractive indices, the light is partially reflected. These reflected beams superimpose and build a characteristic interference spectrum, which is detected with a diode-array. The hybridisation of the ssDNA sample to the surface-bound oligomer results in a change in the optical thickness of the latter layer, which causes a change in the reflectance spectrum. The optical thickness is the product of the refractive index and the physical thickness.

As the refractive index n is dependent on the density given from the Clausius Mossotti equation, and the density is dependent on the thermal expansion, the refractive index decreases with increasing temperature, but due to the thermal expansion the physical thickness d increases with increasing temperature. The two contrary temperature-dependent effects result in a rather low influence of temperature on the optical thickness, the product of the refractive index and the physical thickness.

Thus the hybridisation process can be detected time-resolved and label-free at different temperatures as well as during a change of temperature. A scheme of the detection method is shown in Fig. 1. The RIfS set-up is described in detail in the work of Schmitt et al [18].

To enable the temperature to be modified during a measurement, a temperature adjustable flow-cell from ProLiquid, Überlingen, Germany, consisting of a PEEK flow-channel with heating conductors (1NcAc05) on both sides of the transducer and a thermocouple used as temperature sensor, was employed. A scheme of the flow-cell is shown in Fig. 2.

The temperature control unit from ProLiquid, Überlingen, Germany, consists of a PID 2132 temperature control (Eurotherm, Limburg, Germany), a K-type thermocouple (Thermocoax, Stapelfeld, Germany), a RSDA solid state relay (Eurotherm, Limburg, Germany) with direct voltage control, a 10 ms step-response bellow, and a toroidal transformer with 225 VA, 230 V, 2×18 V, with max 6.25 A.

All samples measured with RIfS were handled by the Automated Sample Injection Analyser (ASIA: Ismatec, Wertheim-Mondfeld, Germany), which contains two peristaltic pumps, a six-way valve and an inject-load valve. The sample is driven in continuous flow with 20 µl/min passing the prepared transducer. After hybridisation, the transducer surface is regenerated with 0.25% SDS at pH 2.5.

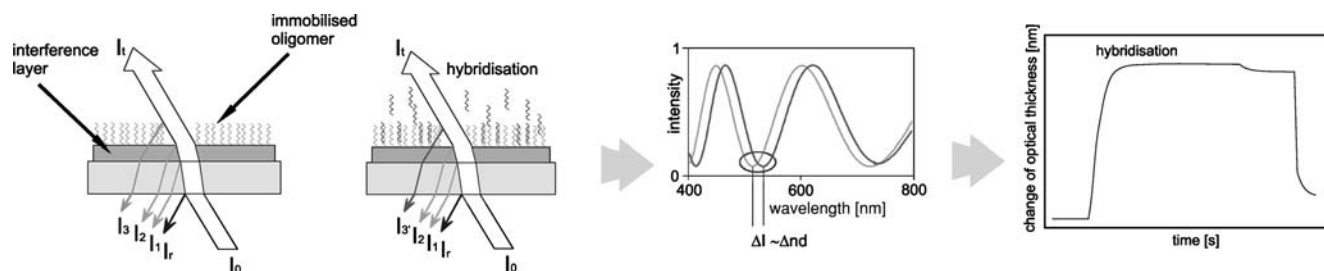


Fig. 1 Scheme of the RIFS detection method. The left part of the scheme shows how the reflected beams superimpose and how the optical thickness of the transducer changes during the hybridisation event on the surface. The right part shows the change in the characteristic interference spectrum and how this shift is transformed into a hybridisation curve

Transducer preparation

The RIFS-transducer chips were cleaned in NaOH for 2 min, then washed with tap water, and their surfaces mechanically cleaned and dried with KIMTECH (Kimberly-Clark, Reigate, UK). After that, the transducer chips were treated with freshly-prepared Piranha solution (mixture of 30% hydrogen peroxide and concentrated sulphuric acid at a ratio of 2:3) for 30 min in an ultrasonic bath to generate silanol groups on the transducer surface. After rinsing with Milli-Q water and drying in a nitrogen stream, the surface was immediately activated by GOPTS for 1 h. Thereafter, the transducers were cleaned with water-free acetone and dried in a nitrogen stream. The coupling of diaminopoly(ethylene glycol) (PEG) (in a solution in dichloromethane, DCM) to this silanized surface was achieved by incubating overnight at 70 °C. After thoroughly rinsing with Milli-Q water and drying in a nitrogen stream, the amino-functionalised surface was treated with glutaric anhydride (GA) in *N,N*-dimethylformamide (DMF) for 6 h to generate carboxylic groups on the surface. The covalent coupling of the amino-terminated single stranded oligomer to this modified transducer surface was achieved using an activated ester, generated by *N,N'*-dicyclohexylcarbodiimide (DCC) in water-free DMF. After incubation for one hour, the transducers were rinsed with DMF before being dried in a nitrogen stream. After that, the transducers were processed immediately. To obtain a high-density DNA-coated transducer with minimal volumes of substances, the single stranded oligomer was spotted on the activated transducer surface using a piezo-based microdosing

system (MicroDrop, Norderstedt, Germany) [19]. These transducers carry a homogenous, high density layer of immobilised single stranded oligomers [20], and they can therefore serve as sensor surface.

Measurement procedure

All measurements were performed in the aforementioned flow-cell. Hybridisation of the nucleic acids was performed in a range of 25–80 °C, depending on the expected melting temperature. For the hybridisation measurements, 1 nmol/ml of ssDNA sample was injected at a constant flow of 20 μ l/min, and after reaching the equilibrium state the surface was regenerated with 0.25% SDS, pH 2.5. For each oligomer and each temperature, the measurements were performed three times. To ensure that thermal equilibrium was maintained in the flow cell and the transducer, a measurement delay of 15 min was set after each temperature increase. To obtain the melting curves of the oligomers, the equilibrium loading of the sensor versus the temperature was drawn, and a sigmoidal fit was performed. The melting point of oligomers is defined as the temperature at which half of the ssDNA is hybridised to its complementary oligomer (immobilised on the surface); this is the inflection point of the sigmoidal melting curve.

For the measurement of the continuous melting curve, a time-dependent temperature gradient was used during the measurement, the hybridisation of 1 nmol/ml ssDNA was performed at room temperature, and after obtaining the equilibrium loading, the flow cell was heated with a temperature program where the linear increase of temperature was 0.5 °C/min, with a constant buffer flow rate, 20 μ l/min. This measurement was repeated without hybridisation at the same conditions using the same temperature program, and then this was subtracted from the hybridisation measurement to eliminate the rather low temperature influence.

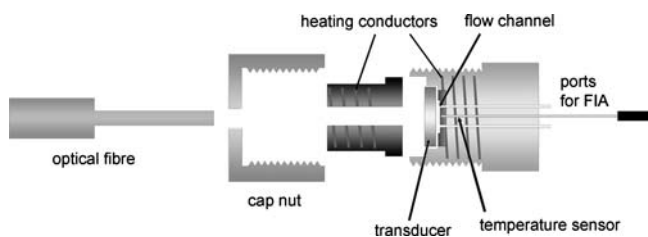


Fig. 2 Design of the temperature-adjustable RIFS flow cell

Results and discussion

For the DNA/DNA hybridisation, the immobilised single strand FBMG1 was hybridised with its complementary ssDNA at the temperatures 25, 30, 35, 40, 50 and 60 °C. The resulting hybridisation curves are shown in Fig. 3.

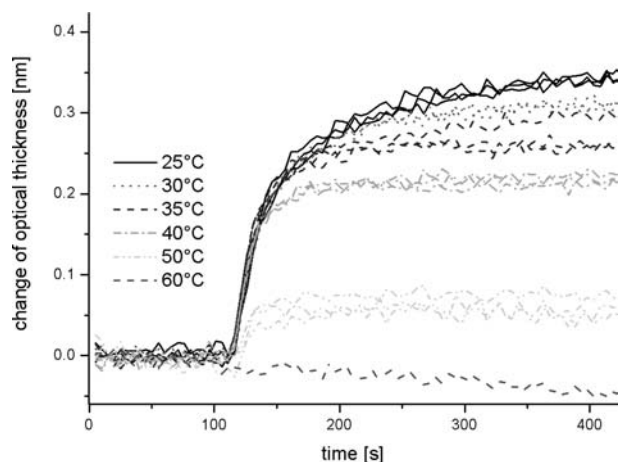


Fig. 3 Hybridisation curves of the complementary FBMG1 DNA with the surface-immobilised DNA FBMG1 at different temperatures

The melting temperature of the DNA/DNA was calculated from the melting curve (Fig. 4) to 42.25 °C. The theoretically-calculated melting point is 43.14 °C. The calculation was performed using Primer Premier (Premier Biosoft International, Palo Alto, CA, USA).

For the PNA/DNA hybridisation, the peptide nucleic acid with the FBMG1 sequence was immobilised on the transducer surface. The complementary ssDNA sample was used as before and the measurements were performed at the temperatures 30, 40, 45, 50, 55, 60, 65 and 70 °C. The melting temperature of the PNA/DNA hybridisation was calculated from the melting (Fig. 4) curve to be 54.84 °C.

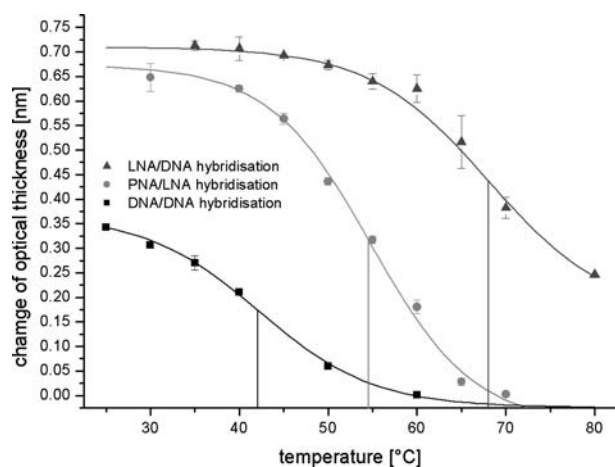


Fig. 4 Melting curves of the LNA/DNA, PNA/DNA and DNA/DNA duplexes. Single stranded LNA, PNA and DNA with the sequence FBMG1 were immobilised on the transducer surface and hybridisations were performed at different temperatures with the complementary ssDNA. To calculate the melting curves, a sigmoidal fit was accomplished using the data evaluation software Origin (OriginLab Cooperation, Northampton, MA, USA). The melting temperature was calculated from the inflection point of the fit function

For the LNA/DNA hybridisation, LNA with the FBMG1 sequence was immobilised on the surface and hybridised with the complementary ssDNA sample at the temperatures 35, 40, 45, 50, 55, 60, 65, 70 and 80 °C. The stability of the PEG layer above the preparation temperature (70 °C) was rather weak, and so it was only possible to get a single measurement at 80 °C within this test series. The melting temperature was calculated from the melting curve (Fig. 4) to be 68.04 °C.

As expected, the DNA/DNA duplex has the lowest melting temperature. The stability of the PNA/DNA duplex is significantly higher due to the lack of the repulsion caused by the negatively-charged backbone in DNA [21]; we found it to have a melting point that was 12.59 °C higher than the melting point of the DNA/DNA duplex. The duplex with the highest stability of the tested oligomers is the LNA/DNA duplex, with a melting point of 54.84 °C, which is 13.2 °C higher than the PNA/DNA duplex and 25.79 °C higher than the melting temperature of the DNA/DNA duplex. This high stability of the LNA/DNA duplex is caused by a local geometry change in the phosphate backbone, which is in good agreement with the literature [22]. All melting curves are shown in Fig. 4, and the melting temperatures are given in Table 1.

Based on these results, we investigated the discrimination of the SNPs with each of the above-mentioned surface-bound nucleic acid derivatives. Therefore, we compared the complementary ssDNA sample (FBMG1) with two mutations, one with one SNP (FBMG2) and one with two SNPs (FBMG3).

At room temperature it was not possible to obtain melting curves for the two mutations with the surface-bound FBMG1 DNA, because the melting temperature is so low that the grade of hybridisation at room temperature is too small to achieve a detectable equilibrium loading. Since the flow cell is not designed for cooling, it does not allow us to measure below room temperature. Therefore, we could not determine the melting temperatures of these SNP duplexes.

For the immobilised PNA, hybridisation with the ssDNA sample FBMG2 was performed at 25, 30, 35, 40, 45 and 50 °C. The melting temperature for the PNA/DNA-FBMG2 duplex was calculated from the melting curve to be 39.08 °C. For the second mutation with two SNPs, it also was not possible to determine a melting temperature, due to the same reasons mentioned before.

It was, however, possible to measure melting curves for both mutations with the surface-immobilised LNA. The LNA-FBMG1 hybridisation with the ssDNA sample FBMG2 was performed at 30, 40, 50, 60 and 70 °C. The melting temperature for the LNA/DNA-FBMG2 duplex was calculated from the melting curve (Fig. 5) to be 57.17 °C. The hybridisation with the ssDNA sample FBMG3 with two SNPs was performed at 30, 40, 50 and 60 °C. The melting temperature for the LNA/DNA-FBMG3 duplex was calculated from the melting curve (Fig. 5) to be 43.54 °C.

Table 1 Measured melting temperatures of the oligomer duplexes

	Immobilised DNA (FBMG1)	Immobilised PNA (FBMG1)	Immobilised LNA (FBMG1)
Complementary DNA (no SNP)	42.25 °C	54.84 °C	68.04 °C
FBMG2 DNA (one SNP)	–	39.08 °C	57.17 °C
FBMG3 DNA (two SNPs)	–	–	43.54 °C

Melting temperatures for all three DNA samples—FBMG1 complementary strand, FBMG2 with one SNP, and FBMG3 with two SNPs—were only obtained with the surface-immobilised LNA. The melting curves are shown in Fig. 5.

Only FBMG2 with one SNP could be detected with the immobilised PNA. With the immobilised DNA, it was not possible to detect any SNP using this set-up, because the melting temperatures are supposed to be below room temperature. The resulting melting temperatures are listed in Table 1.

In addition to the calculated melting curves from the equilibrium loadings at discrete temperatures, we measured continuous melting curves by varying the temperature during the time-resolved RIfS measurements, utilising the thermal stability of the RIfS-method. The immobilised ssDNA-FBMG1 was hybridised with its complementary ssDNA at room temperature. After reaching the equilibrium loading, the temperature was increased during the measurement at a constant rate of 0.5 °C/min. This was performed at a flow of 20 µl buffer per minute. The same measurement was done without hybridisation and was used as reference. After subtraction of the reference from the hybridisation measurement, a melting curve was obtained, and the melting temperature was calculated to 43.17 °C.

This value correlates very well with the melting temperature of 42.25 °C derived from a melting curve obtained from discrete temperature measurements carried

out before, and with the theoretically-calculated melting temperature of 43.14 °C. The use of a time-dependent temperature gradient during RIfS measurement is therefore shown to be a fast and economic method of obtaining melting temperatures from just one measurement.

Conclusion

In the present work we demonstrated that RIfS is an excellent label-free method that can be applied to temperature-dependent DNA hybridisation measurements. It is possible to obtain melting curves for DNA/DNA, PNA/DNA and LNA/DNA duplexes and to determine their melting temperatures. On the one hand, this can be achieved by measuring the equilibrium loading at different discrete temperatures. Changes between these different temperatures need only minimal technical effort, as mentioned before, and can be performed very quickly. On the other hand, a continuous melting curve can be measured by applying a time-dependent temperature gradient during RIfS measurement, which is only possible due to the stability of this method towards temperature changes. Moreover, the RIfS system can be easily modified for use with a two-dimensional format, like the microtiter plate [23]. This gives the label-free RIfS method the potential to be a promising tool, not only for SNP analysis, but also for any type of tem-

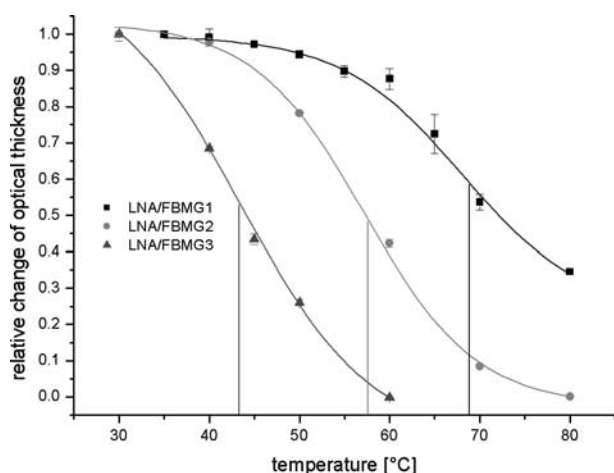


Fig. 5 Melting curves of the two DNA mutations FBMG2 (with one SNP) and FBMG3 (with two SNPs) with surface-immobilised FBMG1 LNA in comparison to the LNA/DNA duplex with the complementary FBMG1 DNA. The change in the optical thickness was referenced to the equilibrium loading at 30 °C

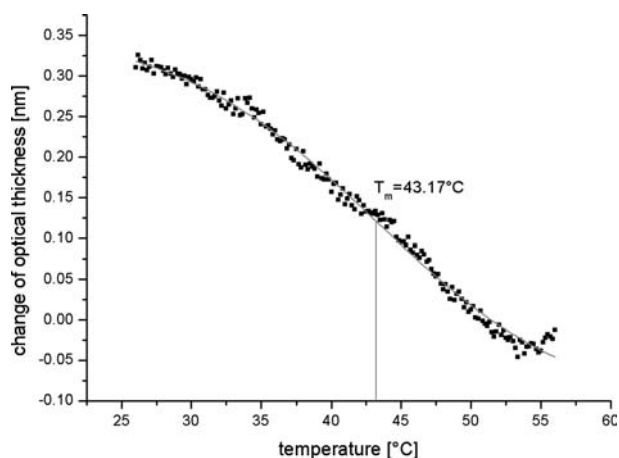


Fig. 6 Continuous melting curve of the DNA/DNA duplex, obtained using a time-dependent temperature gradient with 0.5 °C/min after hybridising the surface-immobilised ssDNA FBMG1 with the complementary ssDNA sample at room temperature. The melting temperature was calculated from the sigmoidal fit

perature-dependent hybridisation experiment. The temperature independence of RfS, and the ability to handle fast temperature changes are two of its major advantages over other label-free detection methods, like SPR or acoustic waveguides [24].

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