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Detection and kinetic studies of triplex formation by oligodeoxynucleotides using real-time biomolecular interaction analysis (BIA)

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ABSTRACT

Real-time biomolecular interaction analysis (BIA) has been applied to triplex formation between oligodeoxynucleotides. 5′-Biotinylated oligonucleotides were immobilised on the streptavidin-coated surface of a biosensor chip and subsequently hybridised to their complementary strand. Sequence-specific triplex formation was observed when a suitable third-strand oligopyrimidine was injected over the surface-bound duplex. In addition, a single-stranded oligonucleotide immobilised on the chip surface was able to capture a DNA duplex by triplex recognition. The presence of spermine increases the rate of association between the third strand and immobilised duplex, but at elevated spermine concentrations non-specific association is observed. A preliminary kinetic analysis of triplex formation at pH 5.2 by an 11mer third strand containing thymine, cytosine and uracil is reported. Values for the association and dissociation rate constants were determined to be $(1.9 \pm 0.2) \times 10^3$ M^{-1} s^{-1} and $(8.1 \pm 1.9) \times 10^{-5}$ s^{-1}, respectively.

INTRODUCTION

There is currently a high level of interest in triple helix-forming oligonucleotides. This is largely due to their ability to recognise specific sequences within double-stranded DNA and their resultant potential as agents to selectively modulate gene expression. In the antigene strategy, triplex-forming oligonucleotides are targeted to regions of genomic DNA where they may inhibit transcription either by interfering with the binding of transcription factors or by blocking transcriptional initiation or elongation (for a review, see 1). The approach has been successfully demonstrated in cultured cells (2–10). Numerous modifications have been made to the backbones, termini and bases of oligonucleotides (for reviews, see 1 and 11) in order to improve triplex stability, extend the base recognition range, facilitate cellular entry and/or overcome the problem of nuclease degradation. There have been many reports concerning the thermodynamics of triplex formation and the effects of oligonucleotide modifications (see for examples, 12–21), but kinetic data are so far limited. Methods used previously to investigate the kinetics of triplex formation have included a restriction endonuclease protection assay (22), DNase I footprinting (23), a filter binding assay (19,24), fluorescence resonance energy transfer (FRET) (20) and UV optical absorbance techniques which monitor either the hypochromicity (25) or the hysteresis effects (17) which accompany triplex formation. Many of these techniques are indirect and/or require radioactive labelling of oligonucleotides, while UV absorbance methods are only suitable over a limited temperature range. The FRET assay is direct and applicable over a wide range of experimental conditions, but requires time-consuming synthesis and purification of fluorescence-labelled oligonucleotides.

Biomolecular interaction analysis (BIA) is a method by which biomolecular interactions can be monitored in real-time (26–28). The basis for BIA measurements is the optical phenomenon of surface plasmon resonance (SPR), which arises in thin metal films under conditions of total internal reflection (29). The angle of incident light required for SPR to occur depends on a number of factors including the refractive index of the medium close to the metal surface. The BIAcore™ system (Pharmacia Biosensor) uses a sensor chip having a thin gold surface film coated with a dextran matrix on which biomolecules may be immobilised using a variety of coupling chemistries (30). A biomolecular interaction taking place on the surface will lead to a change in refractive index close to the surface and cause a change in the resonance angle. Under the conditions used, this change in resonance angle (expressed in resonance units, RU) is proportional to the surface concentration (mass per unit area) of biomolecules. Interactions occur under conditions of continuous flow over the sensor chip surface.

The results of BIA are presented in the form of a sensorgram which shows the change in resonance units as a function of time. Schematic representations of sensorgrams are shown in Figure 1. Sensorgram A shows relatively slow association of an injected analyte with an immobilised biomolecule, followed by a dissociation phase at the end of the injection. In sensorgram B, the rate of association is faster and the interaction reaches a steady-state phase during the injection, where the rates of association and dissociation of the analyte are equal. The end of the injection is followed by a dissociation phase, which, in this case, is very slow.

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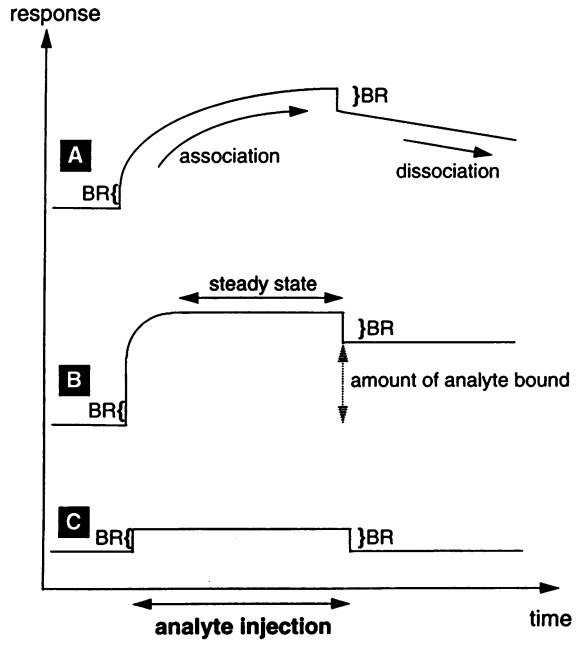

Figure 1. Schematic representations of sensorgrams showing association, steady-state and dissociation phases. BR indicates the bulk response contributions.

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Bt-T30:    5'-Bt-(T)30-3'
T30:       (T)30
A30:       (A)30
T-Con:     T6AT7A2T7AT6
A-Con:     A6TA7T2A7TA6

Bt-AY:     3'-GACTATCTTCTTTTTTCTTCTTCCTAAAGG-Bt-5'
AU:        5'-CTGATAGAAGAAAAAAGAAGAAGGATTTCC-3'
Pso-20:     5'-Pso-TCTTCTTTTTTCTTCTTCCT-3'
Pso-Con:    5'-Pso-CTTTTTCTTTCTTCTTCTTC-3'

Bt-HD1:    5'Bt-CGCTAGAAGAAAGGACG-3'
HD2:         3'-GCGATCTTCTTTCCTGC-5'
HD3:              5'-TCTTCTUTCCT-3'
HD-Con:           5'-CTCTTCUCTTT-3'

Arom30U:   5'-CTGATAGCAGAAAAAAGACGCAGGATTTCC-3'
Arom30Y:   3'-GACTATCGTCTTTTTTCTGCGTCCTAAAGG-5'
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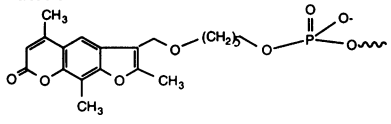
where 5'-Pso is:



and 5'-Bt indicates 5' biotinylated ODNs prepared using biotin phosphoramidite (Cruachem Ltd.).

Figure 2. Sequences of oligodeoxynucleotides used.

Sensorgram C represents an injection of an analyte which does not bind to the immobilised ligand and causes no overall change in response. There may also be a contribution to the resonance signal which is due to the change in refractive index of the bulk solution during the injection, referred to as the bulk response and indicated by BR in Figure 1.

BIA has been used previously to monitor protein–protein and protein–DNA interactions, and DNA–DNA hybridisations (for examples, see 31–34). In this report, we demonstrate that BIA technology can be used to detect specific triplex formation by oligodeoxyribonucleotides (ODNs). We have immobilised 5′-end biotinylated ODNs on the streptavidin-coated surface of a biosensor chip in order to examine triplex formation in three different systems. The first involved the formation of a triplex consisting entirely of T-A·T triplets (we have defined a triplet, X-Y·Z, so that X-Y interact via Hoogsteen-type bonding, and Y·Z is a Watson–Crick base pair). In the second system, the third strand was an ODN containing thymines and cytosines, and linked at its 5′ end to trimethylpsoralen, an intercalating agent. (Psoralens are also photoreactive when exposed to UV light, but the conditions used in these experiments did not permit photoactivation.) The third case involved the formation of a triplex containing T-A·T, C^+-G·C, U-A·T and T-A·U triplets. The sequences of the ODNs used in these experiments are shown in Figure 2.

MATERIALS AND METHODS

Oligonucleotide synthesis

All ODNs were prepared on an Applied Biosystems 391-EP automated DNA synthesizer using standard cyanoethyl phosphoramidite chemistry. The psoralen-linked ODN was prepared using a suitably synthesized phosphoramidite. 5′-Biotinylated ODNs were prepared using biotin CE phosphoramidite (Cruachem Ltd). All non-biotinylated oligonucleotides were purified by ion-exchange HPLC on a Protein-Pack Q 15HR column (Waters), except for T30, A30, T-Con and A-Con, which were purified by repetitive dialysis using a membrane with a 10 kDa molecular weight cut-off (the molecular weights of these ODNs were ~11 kDa). Biotinylated ODNs were washed with butan-1-ol to remove free biotin. Full-length sequences but not failure sequences were 5′-biotinylated during oligonucleotide synthesis and, since only biotinylated ODNs can bind to the streptavidin surface, no further purification was considered necessary.

Biosensor instrument

A BIAcore™ instrument (Pharmacia Biosensor) was used in all experiments. Sensor chips SA5 (research grade), pre-coated with streptavidin (Pharmacia Biosensor) were used for all experiments. Running buffers for each system are shown in Table 1. All experiments were carried out at room temperature (~18°C).

Capture of biotinylated ODNs and detection of triplex formation

Stock solutions of 5′-biotinylated ODNs, Bt-T30, Bt-AY and Bt-HD1, with optical absorbances at 260 nm were 4.752, 6.278 and 3.757, respectively, were prepared (these absorbances would correspond to 20 μM solutions if the oligonucleotides had been fully purified). The stock solutions were diluted 1:10 in a suitable buffer (the same as the running buffer) and 30 μl was injected over the chip surface. The flow rate was either 5 or 10 μl/min.

Capture of the biotinylated ODN was followed by a 30 μl injection of its complementary strand in the same buffer. Thirty microlitres of a suitable control third strand was then injected, followed by a 30 μl injection of the triplex-forming third strand ODN, both in appropriate buffers (see Table 1). Control oligonucleotides were designed by either scrambling the sequence of the

triplex-forming ODN (Pso-Con and HD-Con) or by substituting bases to introduce mismatches unfavourable for triplex formation (i.e. T-Con).

Table 1. Buffers used for each system. The running and sample buffers were identical.

System	Buffer	Buffer constituents
Bt-T30/A30/T30	TAM pH 7.0	40 mM Tris acetate pH 7/ 5 mM $MgCl_2$
Bt-AY/AU/Pso-20	TAM pH 7.0/ 10 µM spermine	40 mM Tris acetate pH 7/ 5 mM $MgCl_2$ / 10µM spermine
Bt-HD1/HD2/HD3	TAE /10 µM spermine pH 5.2	40 mM Tris acetate pH 5.2/ 5 mM EDTA/10 µM spermine tetrachloride

Capture of A30·T30 duplex by surface-bound Bt-T30

Bt-T30 was bound to the chip surface as described. Duplexes A30·T30, A-Con·T-Con and Arom30U·Arom30Y were annealed prior to injection by heating at 95°C for 5 min followed by slow cooling to room temperature. A 30 µl sample of each in TAM pH 7 buffer was injected over the surface.

Regeneration of surface-bound duplex

Subsequent to triplex formation, a 10 µl injection of 10 mM aqueous sodium hydroxide was found to break the Hoogsteen hydrogen bonds between the third strand and the purine strand of the duplex, and to partially denature the duplex. Regeneration of immobilised duplex was therefore carried out by a 10 µl injection of 10 mM NaOH followed by a 30 µl injection of 2 µM complementary strand (A30, AU or HD2).

Spermine dependence of association

Samples (20 µl) of 2 µM T30 supplemented with 0, 1, 10, 100 or 1000 µM spermine^{4+} were injected over immobilised Bt-T30·A30 duplex. The running and sample buffers were TAM pH 7, and the flow rate was 2 µl/min. The surface was regenerated before each injection as described above. The experiment was repeated with T-Con and Arom30U as third strand.

Kinetic analysis of triplex formation by HD3

Samples (30 µl) of 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 10.0 or 25.0 µM HD3 were injected over immobilised Bt-HD1·HD2 duplex. At the end of the injection, dissociation of the complex was monitored for 15 min. The running and sample buffers were TAE pH 5.2/10 µM spermine, and the flow rate was 2 µl/min. The surface was regenerated before each injection as described above.

Association and dissociation rate constants, k_a and k_d, were determined from each sensorgram using BIAevaluation software (Pharmacia Biosensor) which fits experimental data to ideal curves by non-linear curve fitting methods (35). The model used assumes that an injected analyte, A, binds to a single site on an immobilised ligand, B, and that the kinetics are first-order with respect to A and B, and not limited by mass transport effects. The

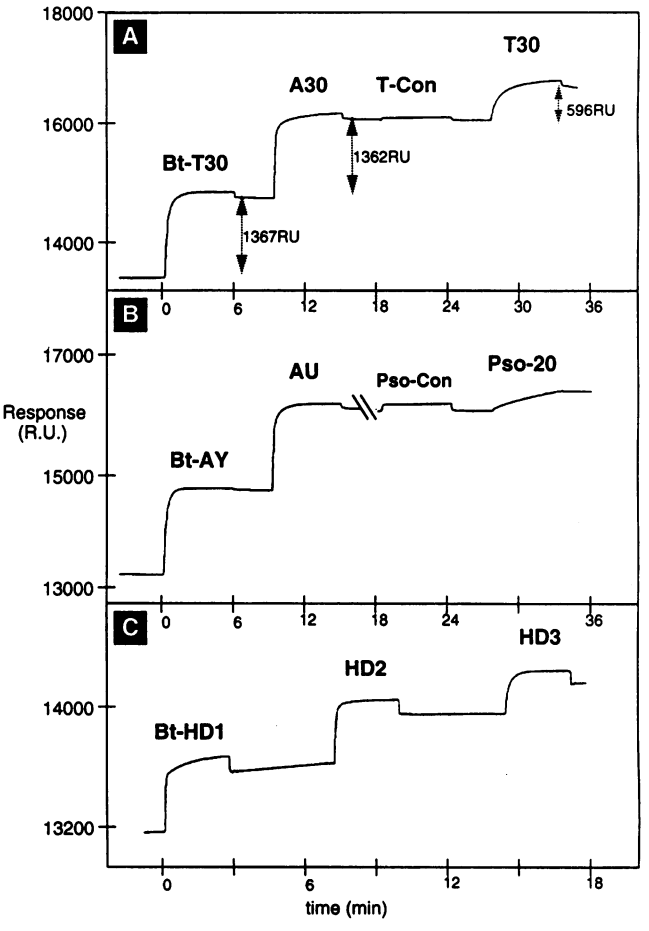


Figure 3. Detection of triplex formation using BIAcore. Oligonucleotide sequences are shown in Figure 2; running/sample buffers were as shown in Table 1. The concentrations of the injected samples were 2 µM (A30, AU, HD2, T30 and Pso-20) or 20 µM (HD3). Flow rates were 5 µl min^{-1} (**A** and **B**) or 10 µl min^{-1} (**C**).

dissociation rate constant, k_d, is determined by fitting the dissociation data to the equation:

$$R = R_0 e^{-kd(t - t0)}$$

where R is the response, t is the time in seconds, and R_0 and t_0 are respectively the response and time at the start of the dissociation. The association rate constant, k_a, and the steady state response level, R_{eq}, are calculated by fitting the association data, using the value of k_d determined previously, to the equation:

$$R = R_{eq}(1 - e^{-(kaC + kd)(t - t0)})$$

where C is the molar concentration of injected analyte and t_0 is the start time for association. The non-linear analysis also includes terms to account for baseline drift in response and bulk refractive index change upon injection of an analyte.

RESULTS AND DISCUSSION

Detection of triplex formation

Figure 3 shows typical sensorgrams representing the formation of triplex in the three systems described. An injection of Bt-T30 (sensorgram A) over the surface caused an increase in response

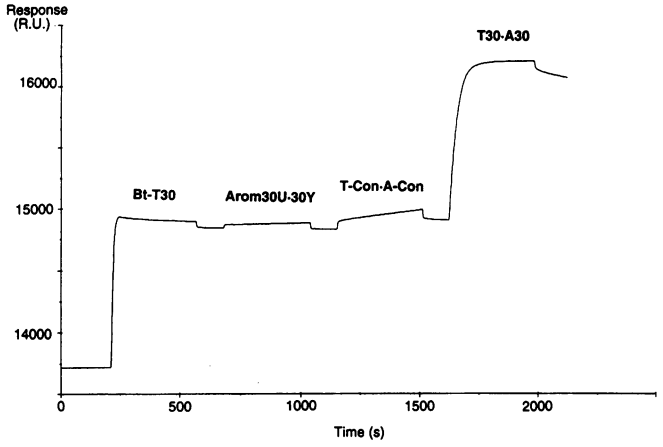


Figure 4. Capture of duplexes by immobilised Bt-T30. The concentration of each injected duplex samples was 2 μM.

of 1367 RU, corresponding to the capture of the biotinylated ODN. This was followed by injection of A30 which gave a further increase of 1362 RU, corresponding to duplex formation. A control ODN, T-Con, was subsequently injected over the immobilised duplex and caused no overall change in response. Finally, T30 was injected over the immobilised duplex and an increase in response of 596 RU, corresponding to triplex formation, was observed. Similar sensorgrams were produced for the other two systems described. To ensure that the observed interactions were specific, a number of controls were performed. Each of the non-biotinylated ODNs was injected in turn over the streptavidin-coated surface of a biosensor chip and none were found to bind.

No interaction was detected between the third strands (T30, Pso-20 and HD3) and their corresponding surface-bound biotinylated ODNs (Bt-T30, Bt-AY and Bt-HD1, respectively). A purine-rich 30mer of unrelated sequence (Arom30U) and a control third strand (T-con, Pso-Con or HD-Con) were injected in turn over surface-bound duplex in each of the three systems and showed no significant binding. This behaviour was reproducible during several experiments.

Capture of duplex

Figure 4 shows the capture of T30·A30 duplex by immobilised Bt-T30. An unrelated duplex, Arom30U·Arom30Y, did not bind and a further duplex, T-Con·A-Con, which could form a triplex containing 4 T·T·A mismatches showed very weak binding to Bt-T30. The observed increase in response on injection of A30·T30 was unlikely to be due to duplex formation between the immobilised strand and any unannealed or displaced A30, since no binding was detected when duplex T-Con·A-Con was injected over surface-bound Bt-T-Con (not shown).

Spermine dependence of triplex association

Figure 5 shows the association of T30, T-Con and Arom30U with immobilised Bt-T30·A30 in the absence of spermine and at 10 μM and 1 mM spermine. It was clear that the presence of spermine increased the rate of triplex association. In the presence of 1 mM spermine, a high level of association was observed for all three third strands. One μM spermine had little effect and

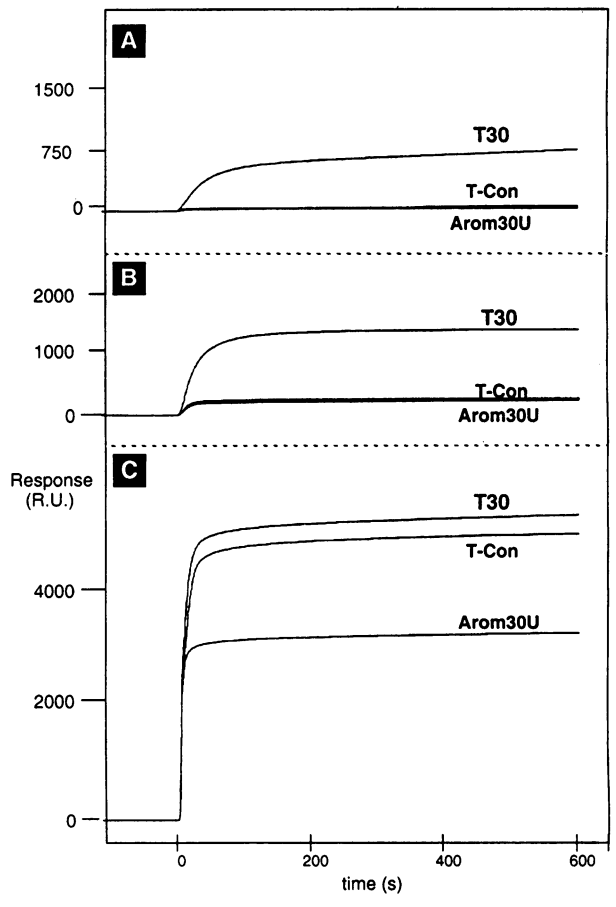


Figure 5. Association of T30, T-Con and Arom-30U with immobilised Bt-T30·A30 in the presence of (**A**) 0 μM, (**B**) 10 μM or (**C**) 1 mM spermine. The response is realative to immobilised duplex. The bulk responses, estimated by injecting the sample over a streptavidin-coated surface, were 40, 60 and 925 RU for 0 μM, 10 μM and 1 mM, respectively.

at 100 μM spermine some non-specific interaction was observed (not shown). This reduced specificity of binding at higher spermine concentrations demonstrates the importance of optimising conditions so that only the binding of interest occurs.

Kinetics of Bt-HD1-HD2·HD3 triplex formation

It was decided that the HD1-HD2·HD3 system would be used to obtain quantitative kinetic data for triplex formation. The T30-A30·T30 system was not considered to be the best model for quantitative analysis due to several factors which made it difficult to ensure that the amount of immobilised available Bt-T30·A30 duplex was constant after each regeneration. First, it was possible, although unlikely given the relatively low surface concentration of biomolecules, that any single-stranded Bt-T30 immobilised on the surface could form a triplex with adjacent immobilised Bt-T30·A30 duplex. Secondly, we found that an excess of A30 injected over immobilised Bt-T30 caused an increase in response slightly greater than that which could be accounted for on the basis of duplex formation alone. This may have been due to antiparallel triplex formation to give a small amount of A30-A30·T30 triplex. A poly r(A-A·U) triplex has previously been reported and forms when the poly r(A) strands are between

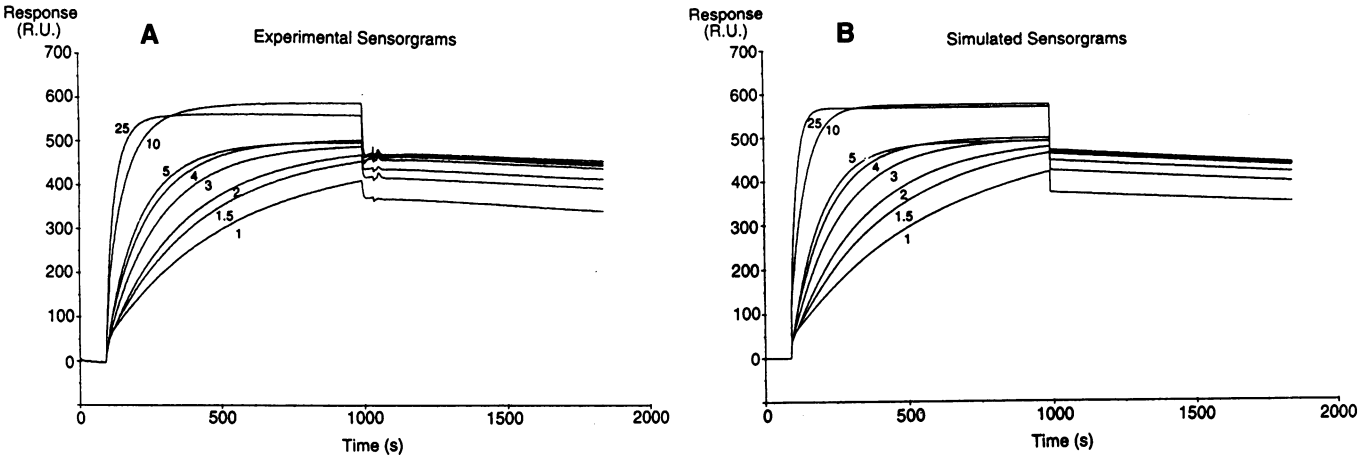


Figure 6. (**A**) Binding of HD3 to immobilised Bt-HD1·HD2. The response is relative to the immobilised duplex. (**B**) Simulated sensorgrams using BIAsimulation software. Values of 1.9×10^3 M^{-1} s^{-1} and 8.1×10^{-5} s^{-1} were used for the association (k_a) and dissociation (k_d) rate constants, respectively. Bulk responses were simulated using values calculated by BIAevaluation software from the experimental sensorgrams. A value of 470 RU was used for R_{max}, the response at saturation of HD3 binding.

28 and 150 bases long (36). An intramolecular triplex containing only d(A-A·T) triplets has also been reported (37). The data for the binding of the psoralen-linked ODN, Pso-20, did not show a good fit to the simple kinetic model used. It is possible that the rate of triplex formation in this case is limited by two steps: intercalation of the psoralen and nucleation of the triplex.

Rate constants for dissociation and association of the third strand, HD-3, to the pre-formed duplex, Bt-HD1·HD2 were calculated to be $k_a = (1.9 \pm 0.2) \times 10^3$ M^{-1} s^{-1} and $k_d = (8.1 \pm 1.9) \times 10^{-5}$ s^{-1} under the conditions used (40 mM Tris acetate pH 5.2, 5 mM EDTA and 10 μM spermine). These values were obtained by taking an average of the values calculated from six sample concentrations (1, 1.5, 2, 3, 4 and 5 μM). The experimental data showed good fits to the model used. χ^2 is a measure of the goodness of fit between the experimental data and the ideal curves, and should be the same order of magnitude as the noise level (typically <2 RU). The χ^2 values for the data used to calculate k_d are <0.5, and ≤ 0.8 for those used to calculate k_a.

BIAsimulation software (Pharmacia Biosensor) was used to generate ideal sensorgrams using the values for k_a and k_d given above. Good correlation is observed between the experimental and theoretical sensorgrams over the entire range of analyte concentrations used, as shown in Figure 6. The BIAsimulation program was also used to determine that, under the conditions used, the interaction was not limited by mass transport of analyte to the surface. These preliminary data do not take into account either dissociation of the duplex or the immobilised complex from the surface, which are likely to be slow compared to triplex dissociation. These factors will be considered elsewhere in a fuller analysis.

It is not clear how the surface immobilisation of one component of a reaction will affect the kinetics of the reaction, or whether the rate constants obtained will differ from a reaction with all components free in solution. The kinetics of triplex formation will depend on many factors, including pH, temperature, ionic conditions and length and sequence of the oligonucleotides used. Previous reports of kinetic constants have used different experimental conditions to those used in this case and therefore a direct comparison with our results is not possible. However, most of these (17,19,20,22,25) provide values for k_a in the range of 10^3 M^{-1} s^{-1}, about three orders of magnitude slower than duplex formation.

CONCLUSIONS

The ability of an oligonucleotide to efficiently inhibit the binding of transcription factors or block RNA polymerase is likely to depend on its ability to compete for the DNA target and on the lifetime of the triplex formed. Rate constants will therefore undoubtedly be important factors affecting the efficacy of oligonucleotides as antigene agents.

A number of methods have been employed to measure rate constants of triplex formation. These are often indirect and may be limited by poor time resolution and/or low sensitivity. Most of the techniques require radioactive or fluorescent labelling and purification of oligonucleotides. A technique which can directly monitor triplex formation with a high sensitivity, over a wide range of conditions and which does not require time-consuming labelling and purification procedures, is therefore highly desirable.

Our results have shown that biomolecular interaction analysis can be used to monitor triplex formation in real time, using a variety of experimental conditions, and to easily obtain kinetic data for both association and dissociation. Furthermore, only one of the three strands needs to be labelled with biotin and minimal purification is required. It should be stressed, however, that kinetic data obtained from BIA experiments may not necessarily correspond to values determined for the equivalent oligonucleotides in free solution. Thus, for example, immobilisation of one or more of the oligonucleotide component strands may (i) reduce the inherent molecular mobility for subsequent hybridisation events, and/or (ii) introduce secondary factors involving surface concentration or aggregation phenomena. Further experiments are required to establish whether such factors are important, but the BIA technique will nevertheless be particularly suitable for rapid comparative studies.

On the basis of the present results, we predict that BIA will prove to be a useful technique for biophysical studies of oligonucleotide-

directed triplex formation, allowing rapid comparison of third-strand binding affinities and providing a method for obtaining important kinetic data for both unmodified and modified oligonucleotides.

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