



Chapter 4

Biosensor-Surface Plasmon Resonance: Label-Free Method for Investigation of Small Molecule-Quadruplex Nucleic Acid Interactions

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Abstract

Biosensor-surface plasmon resonance (SPR) technology is now well established as a quantitative approach for the study of nucleic acid interactions in real time, without the need for labeling any components of the interaction. The method provides real-time equilibrium and kinetic characterization for quadruplex DNA interactions and requires small amounts of materials and no external probe. A detailed protocol for quadruplex-DNA interaction analyses with a variety of binding molecules using biosensor-SPR methods is presented. Explanations of the SPR method with basic fundamentals for use and analysis of results are described with recommendations on the preparation of the SPR instrument, sensor chips, and samples. Details of experimental design, quantitative and qualitative data analyses, and presentation are described. Some specific examples of small molecule-DNA quadruplex interactions are presented with results evaluated by both kinetic and steady-state SPR methods.

Key words Small molecule-nucleic acid interactions, Kinetics, Steady-state analysis, Mass transfer, Biosensor, Surface plasmon resonance

1 Introduction

Noncanonical DNA structures, formed by conformational rearrangements of genome regions bearing specific base sequences, are a novel mechanism of gene regulation. Four-stranded G-rich helical structures, known as G-quadruplexes (G4), are among the most actively investigated noncanonical DNA arrangements [1–3]. Discovered in 1910 thanks to a curious phenomenon of guanosine gel formation [4], G-quadruplexes are now one of the main structural elements of the genome. Computational predictions using the pattern match $d(G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+})$, where N is any nucleotide base, have identified 375,000 putative quadruplex sequences in the human genome [5, 6]. These noncanonical DNA arrangements are usually found at the ends of human chro-

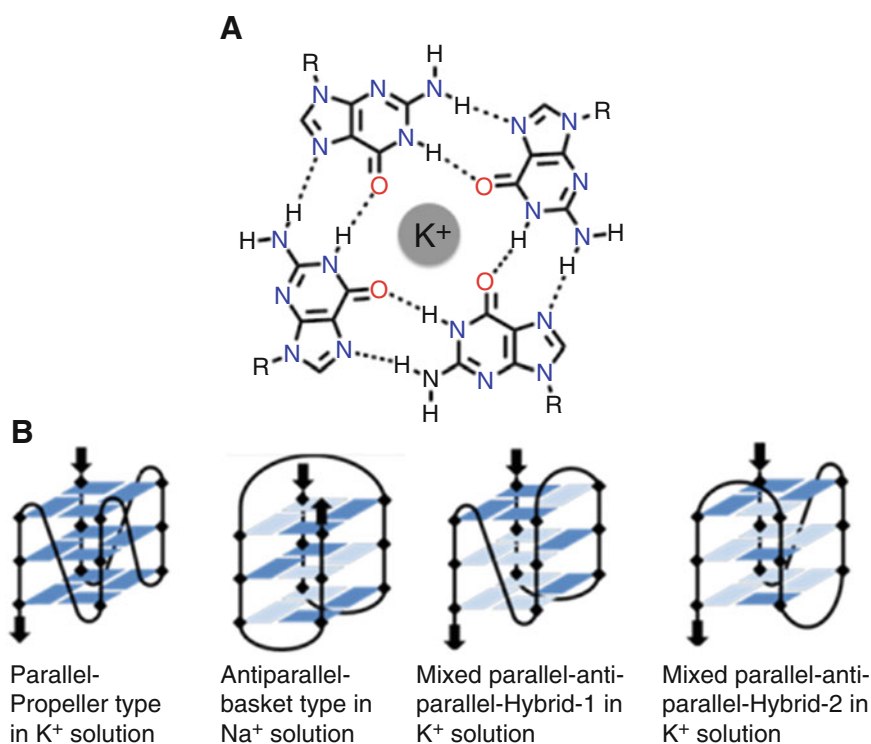


Fig. 1 Schematic outline of (a) a G-quadruplex tetrad and (b) folding topologies of DNA G-quadruplexes in presence of monovalent cations

mosomes (telomeric G-quadruplexes) [7, 8] as well as at promoter regions of many oncogenes, where there is a high population of guanine-rich sequences [9–12]. In physiological conditions, guanine bases can associate through a network of Hoogsteen bonds to form planar arrays known as G-tetrads (Fig. 1). The overlapping of G-tetrads then leads to the formation of more complex arrangements, G-quadruplex structures, which are stabilized by the presence of mono-cations (mainly Na^+ or K^+ , Fig. 1) [13–15].

Interestingly, at telomeres, where there is an equilibrium between the single-stranded repeat of the TTAGGG sequence and its G-quadruplex-folded conformation, G-quadruplex structures are an attractive target for therapeutic intervention. In fact, as G-quadruplex-folded telomeres cannot be recognized by the telomerase enzyme, the stabilization of such arrangements by small molecules can lead to the indirect inhibition of the enzyme activity [16, 17]. This is an attractive approach for the development of new selective anticancer agents as the enzyme is expressed in 85–90% of tumor cells [18–20] while its activity is low, or even absent, in somatic cells [19]. Additionally, as previously mentioned, G-quadruplex structures have also been identified in transcriptional regulatory regions of genes and oncogenes, where they can play a role in

expression control mechanisms [21, 22]. Transcriptional repression of oncogenes through small molecule-driven stabilization of these structures could also be seen as an emerging anticancer strategy.

The thermodynamic signature for the binding of several small molecules to DNA has been extensively investigated as it can help to better understand the features driving the molecular recognition process [23, 24]. In results distinct from those reported for double-stranded DNA, G-quadruplex binders evidenced different binding behavior [25, 26]. A rationale for such differences rests on the polymorphic structure of G-quadruplexes [27–29], which is extremely sensitive to solution composition and G-quadruplex sequence [30–32].

G-quadruplex DNA can assume a number of conformational topologies depending on sequence, length, nature of the monovalent cation, presence of loops, crowding agents, and other environmental factors [29, 33, 34]. An example is represented by the intramolecular telomeric sequence, hTel22, for which a hybrid-type folding appears to be predominant in potassium containing solutions but the coexistence of different conformations in mutual equilibrium has been extensively demonstrated [29, 35–38]. NMR studies reveal that two related telomere sequences in the same experimental conditions assume well-defined but different hybrid forms. Similarly, the 19mer *c-myc* promoter sequence (Fig. 1) can assume a unique parallel conformation [39, 40] while the full-length G-rich sequence of the *c-myc* promoter can assume a number of different G-quadruplex-folded conformations [41].

The broad structural complexity of G-quadruplexes represents a multiplicity of different targets for small molecule binding. For this reason, a detailed characterization of the quantitative behavior of organic small molecules that can selectively interact with G-quadruplexes over duplex DNAs is a key quest [42–46]. Nonselective, off-target binding results not only in a significant reduction of the available compound but also in unwanted toxicity [47, 48]. Therefore, quantitative binding studies can demonstrate enhanced selectivity of specific ligands for a quadruplex sequence and are of fundamental importance in the development of new therapeutic agents.

To establish a basic quantitative characterization of ligand-G4 DNA interactions is essential to determine a set of thermodynamic parameters [49]. In the best case scenario, the binding affinity (the equilibrium constant, K , and Gibbs energy of binding, ΔG), stoichiometry (n , the number of compounds bound to the biopolymer), cooperative effects in binding, and binding kinetics (the rate constants, k , that define the dynamics of the interaction) should be determined. These fundamental parameters are the keys to a molecular understanding of ligand-target interactions and how they translate into a specific phenotype. In order to define such parameters, an accurate method of determining the concentration of

the bound and unbound species is required. Such information should be determined as a function of concentration at equilibrium for accurate K and n , and as a function of time for k determination. For each system then, the question becomes how to accurately determine the necessary concentrations as a function of time and reactant concentration, solution conditions, temperature, etc. Biosensor surface plasmon resonance (SPR) is a label-free method, which is operational down to very low concentration detection [49–51] and can provide all of the desired information. SPR responds to the refractive index or mass changes at the biospecific sensor surface upon complex formation [52]. Since the SPR signal responds directly to the amount of compound bound in real time, as versus indirect signals at equilibrium that are obtained for many physical measurements, it provides a very powerful method to study biomolecular interaction thermodynamics and kinetics. The use of the SPR signal and direct mass response to monitor biomolecular reactions also overcomes many difficulties with labeling or characterizing the diverse properties of biomolecules [49–52].

To illustrate the use of the biosensor-SPR method in G-quadruplex DNA-small molecule interactions, a cationic compound with a highly curved-shape and four-linked furan rings, DB1464, (Fig. 3a) was selected. DB1464 was tested for selective G-quadruplex DNA recognition based on the idea that planar and highly curved molecules do not have the proper shape to fit into the minor groove of the double helix [42, 45, 53]. This planar, aromatic, curved shape, however, can effectively stack on the terminal G-tetrads of quadruplexes [54–57]. These features are an advantage in terms of selectivity for a potential G-quadruplex-binding approach and it is reinforced by the fact that G-quadruplex structures are characterized by the presence of external planar tetrads available for π - π stacking interactions with ligands. Interaction of this compound with distinct DNA arrangements was evaluated using two sequences known to fold into a G-quadruplex conformation, the human telomere hTel22 and cMyc19, a 19mer sequence from the proto-oncogene promoter *c-Myc*. Additionally, a double-stranded hairpin sequence, containing AATT-binding sites, was used to compare the selectivity of the compounds between G-quadruplex and duplex DNA.

1.1 Basic Principles of Biosensor-SPR Methods

The results of a biosensor-SPR experiment are typically presented as a series of sensorgrams, in which the SPR-binding signal (response units or RU in Biacore and some other instruments) is shown as a function of time (Fig. 2). For the DNA immobilization with a common dextran sensor surface, covalently attached protein or DNA or capture of a nucleic acid strand with biotin linked to either the 5' or 3' terminus (*see Note 1* for more detail about immobilization). The terminal attachment of biotin, through a flexible linker (Scheme 1), leaves the nucleic acid-binding sites open for complex

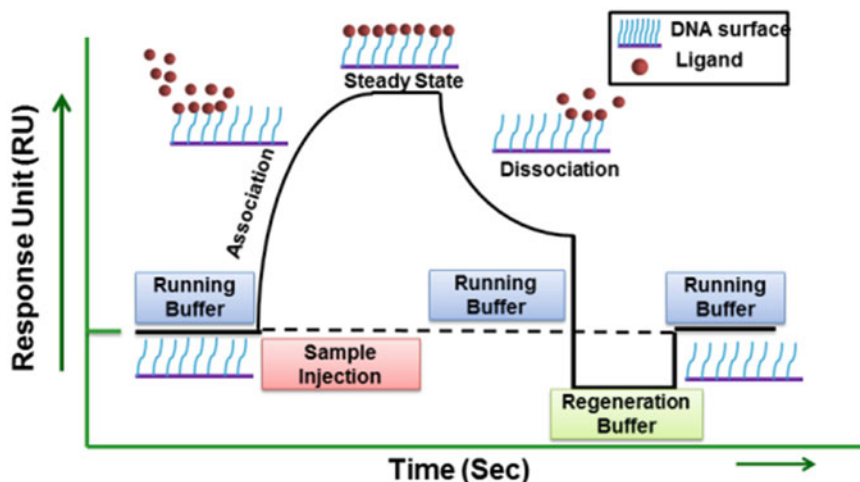
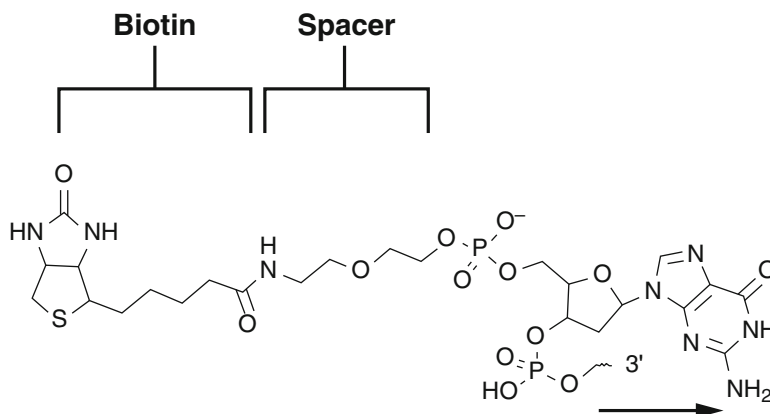


Fig. 2 SPR sensorgram and its components described in steps. (1) Running buffer is injected to stabilize the baseline of the DNA surface; (2) association: ligands are injected over the immobilized DNA, and there is a rise in RU as they bind to the immobilized DNA; (3) bound and unbound ligands in equilibrium at the steady state; (4) dissociation: on injection of running buffer to remove the samples and determine the dissociation constant; (5) injection of regeneration buffer to remove any remaining ligand on the chip; and (6) followed by the running buffer flow to stabilize the baseline for the next ligand injection



Scheme 1 Chemical structure of biotin derivative at 5'-DNA used during immobilization

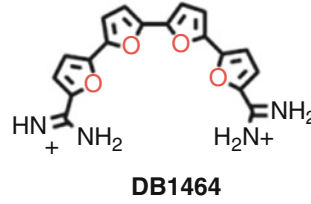
formation. A range of other sensor chip surfaces and immobilization chemistries are also available, and it is generally possible to find an appropriate surface for any biological interaction application (Table 1). In this work and most of our other work, 5'-biotin-labeled DNA sequences (Scheme 1) such as hTel22 and *c-Myc* G-quadruplex-folded sequences and the hairpin-folded duplex control sequence (Fig. 3) were used. In principle 3'-biotin attachment should work in cases where such attachment is an advantage.

A reference baseline is initially established by buffer flow and, secondly, a ligand solution is injected over the surface; the ligand

Table 1
Example of different types of chips (available from GE Healthcare Inc.)

Type	Features	Use
C1	Carboxymethylated, matrix-free surface for covalent immobilization	Need to avoid dextran on the surface for multivalent or very large macromolecules
CM3	Similar properties to sensor chip CM5, suited to large interaction partners and exploratory assay conditions	The interaction partner in solution is very large and exploratory assay conditions
CM7	Similar properties to sensor chip CM5, but for fragment and low molecular weight molecule samples with three times higher capacity	Suitable for work with small molecules and fragment-based screening when achieving the required immobilization level is challenging
SA	Carboxymethylated dextran pre-immobilized with streptavidin for immobilization of biotinylated interaction partners	High binding capacity, reproducibility and chemical resistance give excellent performance over a broad range of application for RNAs and DNAs
HPA	Flat hydrophobic surface consisting of long-chain alkanethiol molecules is attached directly to the gold film. It facilitates the adsorption of lipid monolayers for analysis of interactions involving lipid components	Model membrane systems
L1	Lipophilic groups are covalently attached to carboxymethylated dextran, making the surface suitable for direct attachment of lipid membrane vesicles such as liposomes	High-capacity capture of vesicles and liposomes while maintaining the lipid bilayer
NTA	Carboxymethylated dextran pre-immobilized with nitrilotriacetic acid (NTA). Histidine-tagged molecules are immobilized via Ni ²⁺ /NTA chelation	Capture and immobilization of histidine-tagged molecules via metal chelation
Protein L	Carboxymethylated dextran matrix pre-immobilized with a recombinant protein L for antibodies and antibody fragments containing kappa light chain subtypes (1, 3 and 4) without interfering with its antigen-binding site	Oriented capture of antibody fragments

binding to DNA is monitored by changes in the SPR signal. With sufficient time, a steady-state plateau, where association and dissociation of the ligand are occurring at an equal rate, is established. Finally, buffer flow (without ligand) is reinitiated and the dissociation of the complex is monitored as a function of time (Fig. 2). The above steps are repeated with a series of ligand concentrations and the resulting sensorgrams are fitted to an appropriate binding model as described below.



HTel G4 (22mer): 5'-Biotin-A**GGGTTAGGGTTAGGGTTAGGG**-3'

c-Myc (19mer): 5'-Biotin-A**GGGTGGGGAGGGTGGGG**A-3'

AATT-Hairpin : 5'-Biotin-CCAATT**C** T
 3'-GGT**AAGC** C
 T

Fig. 3 Chemical structure of DB1464 and 5'-biotin-labeled DNA sequences. Gs are taking part in formation of G-tetrads (the compound was supplied by Professor David W. Boykin, GSU)

For a ligand (L) binding to a DNA sequence and forming a single complex (C), the interaction is described by the following equation:



and the equilibrium binding affinity for this interaction is:

$$K_A = \frac{[C]}{[L][\text{DNA}]} = \frac{k_a}{k_d} = \frac{1}{K_D} \quad (2)$$

where $[L]$ is the concentration of the injected ligand, $[\text{DNA}]$ is the concentration of the immobilized DNA not bound to the ligand (free DNA concentration), and $[C]$ is the concentration of the complex; K_A is the equilibrium binding constant, k_a is the association rate constant, and k_d is the dissociation rate constant. For association:

$$\frac{d[C]}{dt} = k_a[L][\text{DNA}] \quad (3)$$

and for dissociation:

$$\frac{d[C]}{dt} = k_d[C] \quad (4)$$

Both the association and dissociation phases of the sensorgram can be simultaneously fit to a desired binding model with several sensorgrams at different ligand concentrations using a global fitting routine [58, 59]. Global fitting allows the most robust determination of kinetic constants (k_a or k_d) and the calculation of

equilibrium constants, K_A or K_D , from the ratio of kinetic constants (Eq. 2). If a steady-state plateau is obtained, the SPR response in the plateau region can be used with the following model to obtain the equilibrium constant:

$$r = \frac{K_A C_{\text{free}}}{1 + K_A C_{\text{free}}} = \frac{RU_{\text{obs}}}{RU_{\text{max}}} \quad (5)$$

(limit of r as $C_{\text{free}} \rightarrow +\infty$)

Note that for Eq. 5, the limit is 1 and this assumes a 1:1 binding model. r represents the moles of bound ligand per mole of DNA total and C_{free} is the free ligand concentration in equilibrium with the complex. RU_{obs} is the observed (experimental) response in the plateau region and RU_{max} is the predicted maximum response for a monomer ligand binding to a DNA site. RU_{max} can be calculated or determined experimentally at the RU for saturation of the DNA-binding sites. In Eq. 5, K_A , r , and RU_{max} are determined by fitting RU_{obs} versus C_{free} . The ratio of observed steady-state response (RU_{obs}) and RU at saturation (RU_{max}) yields the binding stoichiometry.

If the complex dissociates slowly, the surface can be regenerated before the complete dissociation occurs with a solution that causes rapid dissociation of the ligand without irreversible damage to the immobilized DNA [59, 60]. For example, a solution at low or high pH ($\text{pH} \leq 2.5$ or $\text{pH} \geq 10$) can unfold DNA and cause the ligand to completely dissociate. Additional injections of the running buffer (around neutral pH) allow the immobilized DNA to refold and establish a stable baseline. This cycle is repeated with a series of additional ligand concentrations. With a series of sensorgrams generated with a broad range of concentrations, both the kinetics and equilibrium constant can be determined as discussed above.

1.2 Critical Factors for Ligand-DNA Interaction Evaluation by Biosensor-SPR Methods

1.2.1 Concentration Range and Binding Affinity K_D

For accurate determination of equilibrium constants by any method, the selected set of experimental concentrations must provide both free and bound concentrations of reactants. In the biosensor-SPR method with DNA immobilized to the surface, the ligand concentrations should be below and above K_D so that a range of bound fraction of ligand to DNA is obtained. The initial ligand concentrations have less binding to the DNA-binding sites, but as the concentration of ligand injected is increased, the fraction of sites bound on DNA increases and approaches the saturation level. The sensorgrams will have very low binding response at lower ligand concentrations and will approach saturation with higher response at higher ligand concentrations. In this way, a series of sensorgrams with broad ligand concentrations will enable accurate determination of equilibrium constants. If the range of ligand concentrations used is too low or too high, accurate estimation of on-rates and binding constants is not possible. Some preliminary

testing is recommended when the ligand-target approximate K_D is unknown in order to establish an appropriate range of working ligand concentration.

1.2.2 Mass Transport in Association and Rebinding in Dissociation

For the ligand to bind to the DNA (or any target) immobilized to the sensor surface, the sample solution injected over the flow cell surface must be transported from the bulk solution to the immobilized target surface, a phenomenon known as mass transport. This is a diffusion-controlled process, and the transport rate can directly influence the binding kinetics, if the rate occurs slower than the binding reaction. A key requirement for accurate determination of kinetic constants by the SPR method is that the amount of free ligand in the matrix must quickly equilibrate with the flow solution. The equilibration is assisted by using high flow rates. If the association reaction is much faster than mass transport, the observed binding will be limited by the mass transport process. Conversely, if the transport rate is faster than the association rate, the observed binding will represent the true interaction kinetics [52, 61]. Therefore, the mass transport rate is a critical factor that must be considered in biosensor experimental design and in evaluating kinetic constants from biosensor-SPR methods.

Overall, for kinetic measurements, it is generally recommended to use low surface densities of the immobilized DNA and high ligand flow rate to minimize the limitations on binding rates by mass transport processes. In addition, the dissociation phase can be set up for several hours or even longer with Biacore SPR, which allows at least 50% of bound ligand to dissociate, and a reliable kinetic fit can be performed, even with very slow dissociation.

In summary, in biosensor-SPR evaluation of the interaction of ligand-DNA, ligand concentrations, mass transfer, and rebinding have to be evaluated carefully. The incorporation of optimally designed flow cells in the instrument and optimized experimental protocols and sensor chip have qualified biosensor-SPR as an excellent method for quantitative analysis of ligand-DNA interactions, especially for strong binding system.

2 Materials

2.1 Instrumentation

Biacore is a system for real-time label-free biomolecular interactions analysis using surface plasmon resonance technology. A four-channel Biacore instrument, typically a T200 (GE Healthcare Inc.), is recommended for most research studies and it has the best sensitivity of current commercial instruments. Biacore T200 and 2000/3000 instruments use sensor chips with four channels such that three DNAs can be immobilized with one flow cell left blank as a control for bulk refractive index subtraction. With a sensor surface that has covalently attached streptavidin, a nucleic

acid strand with biotin linked to either the 5' or 3' terminus can be captured to create the biospecific surface. The specifications of the instrument have given in the web (<https://www.biacore.com/lifesciences/index.html>).

The materials and procedures presented here are recommended for Biacore instrumentation, but similar reagents and methods are used in other instruments.

2.2 Required Materials for Biacore General Instrument Cleaning and Checking

1. Maintenance chip with a glass flow cell surface (available from GE Healthcare Inc.).
2. 0.5% sodium dodecyl sulfate (SDS, Biacore desorb solution 1).
3. 50 mM glycine pH 9.5 (Biacore desorb solution 2) (*see Note 2*).
4. 1% (v/v) acetic acid solution.
5. 0.2 M sodium bicarbonate solution.
6. 6 M guanidine hydrochloride solution.
7. 10 mM HCl solution (*see Note 3*).
8. HBS-N buffer: 10 mM HEPES pH 7.4, 150 mM NaCl (User prepared or available from GE Healthcare Inc.).
9. BiaTest Solution: (15% (w/w) sucrose in HBS-EP buffer).

2.3 Required Materials for Immobilization of G-Quadruplex DNA on Chip Surface

1. CM5 sensor chip that has been at room temperature for at least 30 min prior to use (sensor chips are available from GE Healthcare Inc.) (*see Note 1*).
2. HBS-EP buffer: 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v polysorbate 20. (User or GE Healthcare Inc.) (Running buffer).
3. Thoroughly filter and degas all solutions. It should be emphasized that the internal microfluidics flow system of the instrument can be damaged by particulate matter present in any solution.
4. 100 mM *N*-hydroxysuccinimide (NHS) freshly prepared in water.
5. 400 mM *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC) freshly prepared in water.
6. 10 mM acetate buffer pH 4.5 (immobilization buffer).
7. 200–400 mg/mL streptavidin prepared in immobilization buffer.
8. 1 M ethanolamine hydrochloride in water pH 8.5 (deactivation solution).
9. Activation buffer (1 M NaCl, 50 mM NaOH).
10. Biotin-labeled nucleic acid solutions (~25 nM of a single strand or hairpin DNA dissolved in HBS-EP buffer).

Table 2
Biacore instrument commands

Biacore control software commands	Function
Desorb	Removes adsorbed materials from the flow system
Sanitize	Removes disinfects from the flow system
Superclean	Washes the flow system and denatures proteins to increase their solubility
Prime	Flushes the flow system with running buffer
Dock	Docks the sensor chip into the instrument
Undock	Undocks the sensor chip from the instrument
Manual run	Allows to control a run interactively
Sample injection	Injects sample

2.4 Sensor Chip Preparation for G-Quadruplex DNA Immobilization

2.4.1 Preparation of Streptavidin Surface on CM5 Chip

1. Dock the CM5 chip and prime with running buffer (*see Note 1*). A manual run is used to establish a stable baseline with a flow rate of 5 $\mu\text{L}/\text{min}$ which is required to make a streptavidin functionalized chip. “Dock” and “Prime” are Biacore software commands that instruct the instrument to carry out specific operations. The commands and operations are listed in Table 2.
2. A solution mixture of 75 μL of NHS and 75 μL of EDC is required to activate the carboxymethyl surface to reactive esters. Note: mix these solutions just prior to injection to get good activation of the surface.
3. Inject NHS/EDC mixture for 10 min (50 μL) to receive optimum amount of reactive esters.
4. By using “Manual Inject” with a flow rate of 5 $\mu\text{L}/\text{min}$ several injections of streptavidin, prepared in immobilization buffer, are injected over all flow cells. The number of RUs immobilized, which is available in real-time readout, are tracked to obtain the desired RU level. This is typically 2500–3000 RUs for a CM5 chip after the injection has been stopped.
5. For the deactivation of remaining ester, 1 M ethanolamine hydrochloride with a flow rate of 5 $\mu\text{L}/\text{min}$ for 10 min is injected in all flow cells.
6. A few primes are necessary to obtain a stable baseline.

2.4.2 G-Quadruplex DNA Immobilization on a Streptavidin Chip

1. A streptavidin-coated sensor chip (chip prepared as outlined above or SA pre-derivatized sensor chips from GE) that has been at room temperature for at least 30 min is required.

2. Biotin-labeled nucleic acid solutions (~25 nM DNA dissolved in HBS-EP buffer is also required (running buffer).
3. Dock the streptavidin-coated chip and by using command manual run, a sensorgram with a 25 $\mu\text{L}/\text{min}$ flow rate is started to check the base line.
4. Activation buffer, 1 M NaCl, 50 mM NaOH is injected for 3 min (75 μL) for a course of five to seven times to remove unbound streptavidin from the sensor chip.
5. To ensure the surface stability, prime with running buffer a few times as necessary.
6. Allow buffer to flow at least 10 min (or until the baseline is stable) before immobilizing the nucleic acids.
7. A new sensorgram with a flow rate of 1 $\mu\text{L}/\text{min}$ is started in a desired flow cell under “flow path” (e.g., flow cell 2, fc2) to immobilize the nucleic acid. Generally, flow cell 1 (fc1) is used as a control and is left blank for subtraction. It is often desirable to immobilize different nucleic acids on the remaining two flow cells (fc3 and fc4).
8. Wait for the baseline to stabilize (which usually takes a few minutes). Use “Manual Inject,” load the injection loop with ~100 μL of a 25 nM nucleic acid solution and inject over the flow cell. Track the number of RUs immobilized and stop the injection after the desired level is reached (typically ~200 RU for 20–30 base-pair DNA for kinetics experiments to minimize mass transport effects).
9. At the end of the injection and after the baseline is stabilized, determine the RUs of the immobilized nucleic acid by using the reference line option. The amount of nucleic acid immobilized is required to determine the theoretical moles of ligand-binding sites for the current flow cell (*see* **Note 4**).
10. Repeat the **items 7 and 8** for immobilization of other DNAs to flow cells fc3 and fc4 separately.
11. After successful DNAs immobilization in all cells (except fc1), immobilization buffer is replaced by experimental buffer and followed by prime the system several times.

2.5 Flow Solutions: Buffers and Samples

2.5.1 General Buffers (See Note 5)

1. (a) 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, (HBS-EP buffer) (GE Healthcare Inc.) (*see* **Note 6**).
- (b) 10 mM MES [2-(*N*-morpholino) ethanesulfonic acid] pH 6.25, 100 mM NaCl, 1 mM EDTA, and P20 (MES10 buffer).
- (c) 10 mM CCL [cacodylic acid] pH 6.25, 100 mM NaCl, 1 mM EDTA, and polysorbate 20, P20, (CCL10 buffer).
- (d) 10 mM Tris [(hydroxymethyl) aminomethane] pH 7.4, 100 mM NaCl, 1 mM EDTA, and polysorbate 20, P20, (Tris10 buffer).

2. The sample solution must be prepared in the same buffer used to establish the baseline (running buffer) (*see Note 7*).
3. The sample concentration to be used depends on the magnitude of the binding constant (K_A). With a single binding site, for example, concentrations at least 10 times above and below $1/K_A$ should be used (i.e., a 100-fold difference between the lowest and highest concentrations). A larger concentration range above and below $1/K_A$ will yield a more reliable/accurate binding curve. For binding constants of 10^7 – 10^9 M^{-1} , as observed with many nucleic acid/small molecule complexes, small molecule concentrations from 0.01 nM to 5 μ M in the flow solution allow accurate determination of binding constants. Injecting samples from low to high concentration is useful as it prevents data artifacts from ligand adsorption (*see Note 8*).
4. Possible problems at high sample concentrations: poor sensorgrams, nonspecific binding, ligand aggregation may be obtained. In this case only the lower concentrations can be used for quantification.

2.5.2 Regeneration Solution (*See Note 9*)

1. Good regeneration conditions help achieving complete removal of binding ligand from the chip surface without the immobilized target degradation. Commonly used regeneration solutions are listed in Table 3. In general, milder conditions are

Table 3
Regeneration solutions

Interaction strength	Acidic	Basic	Hydrophobic	Ionic
Weak	pH > 2.5 10 mM Glycine/ HCl Formic acid	pH < 9 10 mM HEPES/ NaOH	pH < 9 50% ethylene glycol	1 M NaCl
Intermediate	pH 2–2.5 10 mM Glycine/ HCl Formic acid HCl H ₃ PO ₄	pH 9–10 10 mM Glycine/ NaOH NaOH	pH 9–10 50% ethylene glycol	2 M MgCl ₂
Strong	pH < 2 10 mM Glycine/ HCl Formic acid H ₃ PO ₄	pH > 10 NaOH	pH > 10 25–50% ethylene glycol	4 M MgCl ₂ 6 M guanidine-hydrochloride

initially used, while more stringent conditions are applied only as needed. Regeneration solutions for different samples are available in the Biacore website. In our organic small molecules studies a high salt concentration solution or a stronger 10 mM Glycine/HCl (pH 2.5) solution is typically used as an efficient regeneration agent to remove small molecules from the DNA immobilized sensor chip surface.

2. Inject 10–20 μL of regeneration solution twice consecutively at high flow rates to assure efficient regeneration.
3. After injection of the regeneration solution, three 1-min injections of running buffer are recommended to wash off the remaining regeneration solution.
4. At the end of each cycle, a 5 min running buffer flow is also recommended to ensure that the chip surface is re-equilibrated for binding (i.e., the dextran matrix is re-equilibrated with running buffer) and that the baseline is stabilized before the following sample injection.

3 Method

The Biacore software, supplied with the instrument, allows users to write a method or to use a software wizard to set up experiments. Several important factors, such as flow rate, association and dissociation times, injection order, and surface regeneration must be considered while setting up an experiment. A simple method used to collect small molecule binding results on G-quadruplex nucleic acid surfaces is shown below. The structure of the compound (DB1464, prepared in the laboratory of Professor D. W. Boykin at GSU [42]) and the biotin-labeled DNA sequences (hTel22, c-Myc19, AATT-Hairpin) used in this experiment are shown in Fig. 3.

3.1 Data Collection and Processing

1. A four-channel Biacore instrument, typically a T200 (GE Healthcare Inc.), is used in this study.
2. Three 5'-biotin-labeled DNAs are immobilized, each one immobilized in a distinct flow cell of a SA chip, as described in Subheading 2.4, item 2. Approximately the same amount of each DNA oligomer is immobilized on the surface of these flow cells to compare the sensorgram saturation levels directly from stoichiometry differences.
3. 10 mM Tris-HCl buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 0.005% v/v polysorbate 20, pH 7.5) is used as a running buffer.
4. 10 mM Glycine/HCl (pH 2.5) is used as regeneration solution because DB1464 is a strong binder.

5. Serial dilutions (concentration range from 1 nM to 1 μ M) of the DB1464 compound are prepared using the running buffer as the diluent to minimize changes in the refractive index caused by buffer components. The flow rate is set to 50 μ L/min (*see* **Note 10**).
6. A waiting period of 5 min prior to each sample injection cycle is recommended to allow baseline stabilization that is essential for accurate small molecule binding analysis. Several buffer samples are injected at the beginning of each experiment to evaluate if the instrument is performing within specifications. Buffer injections also serve as controls for data processing.
7. Inject 500 μ L (10 min) of each compound concentration and set 600 s (these are varied with different compounds and kinetics) as dissociation time (*see* **Note 11**). Inject samples from low to high concentration to eliminate data artifacts from ligand carry over or contamination of the instrument flow system (*see* **Note 12**).
8. At the end of the dissociation phase, inject two short pulses (typically 30–60 s) of 10 μ L Glycine/HCl (10 mM, pH 2.5), followed by three 1-min injections of running buffers are recommended to reduce the remaining regeneration solution and 5 min running with buffer flowing is also set to ensure that the chip surface is re-equilibrated for binding (*see* Subheading 2.5.2, **item 3**).
9. When the experiment is completed, open the raw data containing the sensorgrams in the BIAevaluation software for data processing (*see* **Note 13**). First, zero the sensorgrams on the y-axis (RU) to allow proper comparison of the responses of each flow cell. Generally, the average of a stable time region of the sensorgram, prior to sample injection, should be selected and set to zero for each sensorgram. Then, zero the x-axis (time) to align the beginnings of the injections with respect to each other [52].
10. Subtract the control flow cell (fc1) sensorgram from the reaction flow cell sensorgrams (i.e., fc2–fc1, fc3–fc1, and fc4–fc1). This removes any bulk shift contribution to the change in RUs.
11. Subtract a buffer injection (the injection with a ligand concentration of zero), or better, an average of several buffer injections from the compound injections (different concentrations) on the same reaction flow cell (*see* **Note 14**). This is known as double subtraction and removes any flow cell specific baseline irregularities [52, 62]. At this point, the data should be of optimum quality and ready for analysis as described below.

3.2 Data Analysis

1. After the data are processed as described, kinetic and/or steady-state analysis is performed. Both kinetic and steady-state fitting can be done in the Biacore software or in other available software packages (such as Scrubber-2,

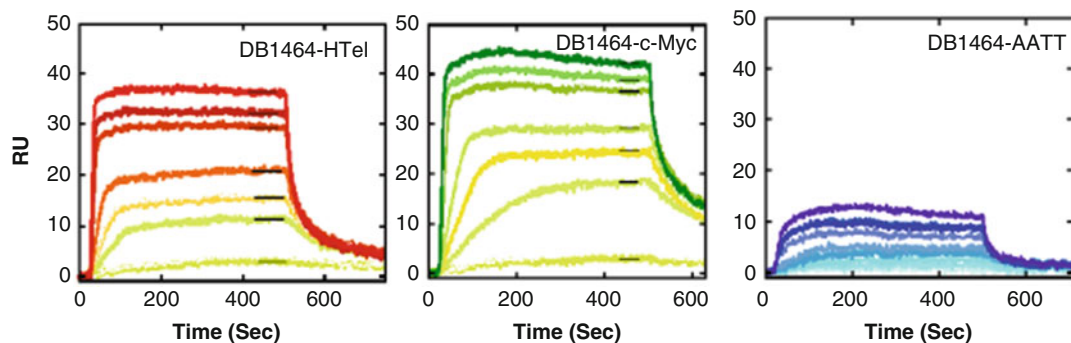


Fig. 4 Respective SPR sensorgrams for the interaction of DB1464 with the hTel22, c-Myc, and AATT hairpin duplex, concentrations of DB1464 from bottom to top are 0–1 μ M

<http://www.biologic.com.au>). As shown in Fig. 4, DB1464 binding reaches a steady-state plateau during the injection period so that a steady analysis can be used to determine the equilibrium constant. In this current experiment the binding rate is not limited by mass transfer and the association and dissociation rate constants can also be determined. The average of the data over a selected time period in the steady-state region of each sensorgram can be obtained, converted to $r = RU/RU_{\max}$ and plotted as a function of compound concentration in the flow solution (*see Note 15*).

2. To obtain the affinity constants, the data were fitted to the following interaction model using *Kaleidagraph* for nonlinear least-squares optimization of the binding parameters:

$$r = \frac{(K_1 C_{\text{free}} + 2K_1 K_2 C_{\text{free}}^2)}{(1 + K_1 C_{\text{free}} + K_1 K_2 C_{\text{free}}^2)} \quad (6)$$

where K_1 and K_2 are equilibrium constants for two types of binding site (for a single site $K_2 = 0$) and C_{free} is the concentration of the compound in equilibrium with the complex and is fixed by the concentration in the flow solution. For a single dominant binding site model, K_2 is equal to zero. Errors in fitting results are less than $\pm 10\%$. As described above, the binding stoichiometry can also be obtained directly from comparing the maximum response with the predicted response per compound.

3. Since in this example, equal amounts of the G-quadruplex forming sequences hTel22 and c-Myc19 and of the control AATT-hairpin duplex were immobilized. The difference in maximum responses among the sets of sensorgrams was immediately observed. The differences in kinetics constants, binding constants, stoichiometry and cooperativity for DB1464 binding to the hTel22 and c-Myc19 G-quadruplex structures and the

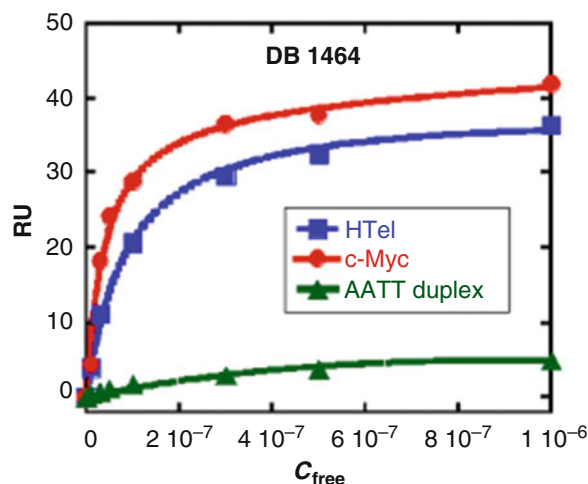


Fig. 5 Comparison of the SPR binding affinity of DB1464 with G-quadruplex sequence, hTel22 (squares), *c-myc* (circles) and AATT hairpin duplex (triangles) DNA sequences. *RU* values from the steady-state region of SPR sensorgrams are plotted against the unbound compound concentration (flow solution). The lines are the best-fit values using appropriate binding models

Table 4
Binding constants obtained by fitting the curves using two-sites binding model

	hTel 22 K_A	<i>c myc</i> 19 K_A	AATT-Hairpin duplex K_A
DB1464	$1.1 \times 10^7 \text{ M}^{-1}$	$2.5 \times 10^7 \text{ M}^{-1}$ $8.0 \times 10^4 \text{ M}^{-1}$	$3.2 \times 10^5 \text{ M}^{-1}$

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G-quadruplex

AATT hairpin duplex, can be obtained as illustrated in Fig. 5. Under these experimental conditions, DB1464 binds with a 1:1 ratio to hTel22 and AATT-Hairpin while for c-Myc19 a secondary weaker binding site has also been observed.

- The steady-state binding responses are fit using a one-site binding equation for hTel22 and AATT-hairpin and a two-site binding model for c-Myc19 (Fig. 5 and Table 4). A comparison of the binding affinities of DB1464 versus the three targets used in the experiment indicates a strong preference for the G-quadruplex DNA conformation, with primary binding constants around 10^7 M^{-1} for both the human telomere and c-Myc while a very weak interaction (>100 -fold less) with duplex DNA is observed in the same environmental conditions (Figs. 4 and 5, Table 4). DB1464 shows the higher affinity to c-Myc19 ($2.5 \times 10^7 \text{ M}^{-1}$) and the interaction with this target occurs with apparently slower rates of association and dissociation.

5. Kinetic analysis using global fitting of SPR data places a great demand on obtaining high-quality data. Experimental design, analysis, and optimization of kinetic studies have been described in detail elsewhere [52]. In general, low surface densities of the immobilized target and high ligand flow rate should be used to minimize the effects of mass transfer. Several criteria must be satisfied when considering if a global kinetic fit is acceptable [52]: (a) within experimental limits, the $RU_{\max}$ is the same as the predicted value or from the steady-state results for one binding molecule; (b) the rate constants are within the range of small molecules; (c) the mass transport constant k_t is in the 10^7 range; (d) $(k_a \times RU_{\max}/k_t) \leq 5$; (e) the half-life $t_{1/2}$ from the dissociation phase of sensorgram is close to the calculated half-life using the fitted value ($t_{1/2} = \ln 2/k_d$), suggesting the mass transport effect is minimized; (f) the residuals are within the instrumental noise and there are no systematic deviations; (g) a low chi-squared value is obtained at convergence.

4 Notes

1. The choice of sensor chip depends on the nature and demands of the application. For general purposes, a Biacore CM5 sensor chip, which carries a hydrophilic matrix of carboxymethylated (CM) dextran covalently attached to the gold surface, can be used. It has a high surface capacity for immobilizing a wide range of ligands from protein to nucleic acids and carbohydrates. For protein-DNA interaction investigation, the Biacore CM4 sensor chip is another good choice because it is similar to sensor chip CM5 but has a lower degree of carboxymethylation (~30% of that of CM5 chip) and charge that helps to reduce nonspecific binding of highly positively charged molecules, such as proteins, to the surface. Streptavidin-coated sensor chip has a surface carrying a dextran matrix to which streptavidin has been covalently attached. Streptavidin has a very high binding affinity for biotin ($K_D \approx 10^{-15}$ M) so that the surface provides a high capture of biotinylated ligands. The streptavidin-coated chip is particularly suited for nucleic acid immobilization since biotin coupling of oligonucleotides at the terminal or the internal positions is a well-established procedure. For some other specialized applications, range of other sensor chips surfaces and immobilization chemistries are also available (Table 1).
2. Maintenance chips are available from GE Healthcare Inc. “Desorb” is a Biacore software command that instructs the instrument to remove adsorbed ligands from the flow system.

A detailed list of commands and operations is shown in Table 2. Make sure that the analysis and sample compartment temperatures are not below 20 °C, since SDS in Desorb solution 1 will precipitate at low temperature.

3. After running the regular Desorb for the additional extensive cleaning, additional super clean method may be used.
4. The amount of DNA to immobilize on the sensor chip depends on the relative molecular weight of the target DNA and of the ligand and on the sensitivity of the biosensor system. Since the SPR response is directly proportional to the mass concentration of material on the surface, the theoretical ligand-binding capacity for a 1:1 interaction of a given surface is relative to the amount of DNA immobilized.
5. The selection of experimental buffer depends on the nature of the ligand and DNA sequence. Salt concentration can be adjusted based on the experimental requirement. With the increase of ionic strength, the binding affinity of positively charged ligands for the negatively charged nucleic acid typically decreases due to charge shielding effects.
6. The amount P20 to be used depends on the system, the instrument and the sensor chip, typically concentrations between 0.05% and 0.005% are used. Other detergents are also used in some cases but for most studies of quadruplexes on a Biacore T200 or X100, P20 at 0.05% is best.
7. If the ligand requires the presence of a small amount of organic solvent (e.g., <5% DMSO) to maintain solubility, a solvent correction needs to be applied to minimize changes in the refractive index caused by the organic content at the chip surface. This has been described in detail by Rich and Myszka [51].
8. If the K_A is unknown, it is necessary to conduct a preliminary experiment using a wide range of compound concentration to obtain an estimate of the K_A . A more focused set of concentrations is then prepared to cover the specific binding range.
9. Regeneration conditions must be harsh enough to break the complex and remove the bound ligand but mild enough to keep the DNA strand intact. It is highly recommended to start with the mildest conditions and short surface contact times since regeneration solutions can cause an undesired effect on DNA or immobilized matrix. A short contact time, 30–60 s, is usually sufficient. Longer exposure to regeneration conditions involves greater risks of loss of binding activity on the surface and often does not lead to improved regeneration.
10. For the steady-state method, equilibrium constants can be obtained even when mass transfer effects dominate the

observed kinetics. Thus, higher flow rates are not required in steady-state experiments, if a clear steady-state plateau is obtained, to determine RU. Higher flow rates ($>50 \mu\text{L}/\text{min}$) are used for kinetic experiments to minimize mass transport effects.

11. A sufficient association phase with a plateau region is needed for steady-state analysis. For the most accurate fitting of the dissociation phase, it is good practice to allow sufficient time for the compound to achieve at least 80% dissociation from the complex.
12. Many organic small molecules are easily adsorbed nonspecifically to the tubing of the injection microfluidics and are slowly released over the course of the experiment. Increasing surfactant concentration might reduce adsorbing to the tubing.
13. Other software programs such as Scrubber 2, CLAMP and GeneData are available for processing Biacore data. The results can also be exported and presented in graphing software such as KaleidaGraph for PC. Although it is useful to experiment with different software packages, BIAevaluation is sufficient for most routine analyses of sensorgram data. For the Biacore T200 user, data processing can be performed automatically using the Biacore T200 evaluation software, which is much more convenient for new users. For processing of Biacore data for large libraries of small molecules, GeneData is a preferred choice.
14. These two data processing steps are referred to as “double referencing.” Typically, multiple buffer injections are performed and averaged before subtraction. In double referencing, plots are made for each flow cell separately overlaying the control flow cell- corrected sensorgrams from the buffer and all sample injections. The buffer sensorgram is then subtracted from the sample sensorgrams. “Double referencing” removes the systematic drifts and shifts in baseline and is helpful to minimize offset artifacts and also to correct the bulk shift that results from slight differences in injection buffer and running buffer.
15. In some cases, at lower concentrations, where the response does not reach the steady-state, the equilibrium responses can be obtained from kinetic fits of the sensorgrams utilizing the known RU_{max} from the higher concentration sensorgrams. This extrapolation method works well with sensorgrams where the observed response is at least 50% of the equilibrium RU.

In conclusion, Biosensor-SPR analysis of nucleic acid targets, either G-quadruplex or dsDNA, or RNA, offers a powerful method of obtaining thermodynamic and kinetics values.

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