Aptasensor Development: Elucidation of Critical Parameters for Optimal Aptamer Performance

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Aptamers are synthetic oligonucleotides specifically selected to bind a certain target. Thanks to their high affinity and sensitivity, aptamers appear as alternative candidates to antibodies for analytical devices and several assays have been reported. However, and contrary to what happens with DNA probes, the aptamers' ability to bind their targets depends on folding and 3-D structure, which may be affected by the incubation conditions and buffer composition. In this report, a systematic evaluation of the parameters with potential effect on the ELONA performance has been carried out. Additionally, diverse ELONA and mixed ELISA/ELONA formats exploiting the thrombin-binding aptamer have been optimized and their efficiencies compared. ELONA results have been confirmed using nuclear magnetic resonance, electrophoresis, and surface plasmon resonance. Our results indicate that parameters such as immobilization strategy, incubation time/temperature, and buffer composition should be optimized for each aptamer as they affect folding and, thus, binding efficiency. Among the studied assays, the mixed ELISA/ELONA sandwich formats showed the lowest limit of detection observed (<1 nM thrombin), while a competition ELONA appeared as the best assay in terms of high sensitivity (1.8 nM) and short assay time (1 h, 30 min). The elucidation of optimal parameters for assay performance reported here clearly indicates that aptamers are unique structures. Formation of the 3-D structures required for target binding is influenced by variable parameters, and unlike DNA/antibody based assays, there are no general recommendations, with each assay requiring individual optimization of parameters.

In 1990, the laboratories of Joyce, Szostak, and Gold[1−3] independently reported on the development of an in vitro selection and amplification technique for the isolation of oligonucleotides able to bind non-nucleic acid targets with high affinity and specificity. This technique was coined as SELEX—*s*ystematic *e*volution of *l*igands by *ex*ponential enrichment—and the resulting oligonucleotides were referred to as aptamers, derived from the latin *aptus*, meaning, "to fit".[2] During the following years, the research community has witnessed the description of an extensive range of aptamers with abilities to bind to small molecules, peptides, proteins, and even whole cells with selectivity, specificity, and affinity equal or superior to those of antibodies.[4−9] In some cases, aptamers have been described to discriminate targets on the basis of subtle structural differences, such as the presence of a methyl or hydroxy group or the D- versus L-enantiomeric configuration of the target.[10]

The advantages of aptamers over alternative approaches include the relatively simple techniques and apparatus required for their isolation, the number of alternative molecules that can be screened, and their chemical simplicity.[11] As aptamers can be evolved to bind tightly and specifically to almost any target, their potential for use in analytical devices, in proteomic microarrays, and as therapeutic agents[4,9,12,13] is immense. As biorecognition elements in analytical devices, aptamers appear as alternative candidates to antibodies (Ab) and antibody fragments (Fab) often offering considerable advantages.[4,9,12,16] Aptamers are chemically produced and can be selected in extreme conditions, permitting the study a broad variety of samples, while Abs in general are only stable in a physiological environment. Simultaneous multi-selection processes can be performed using a single oligonucleotide library. Following selection, aptamers can be easily modified (to incorporate molecular markers, favor oriented immobilization, or increase their stability to nucleases) without affecting their affinity, while chemical modification of Abs often decreases their affinity. Aptamers can routinely be formatted to a reagentless system, in the shape of molecular beacons, as well as other novel strategies under development. The animal-free production of

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Table 1. Summary of the Assays Reported by Other Authors in Which TBA Has Been Used[a]

ref (date)	TBA label/immobil	assay format	detection	binding buffer	reagent concn	assay time	LOD	K_d
34 (2004)	in solution	direct polymeric staining	change in color due to TBA folding	water (± K)	2.9 nmol TBA	few min	10 pM	
26 (2002)	in solution (5′-fluoroph, 3′-quencher) (2 fluoroph)	molecular beacon	change in fluoescence	20 mM Tris 140 mM NaCl 5 mM KCl 1 mM CaCl 1 mM MgCl 5% glycerol	(l00 μL) 1 μM TBA		373 pM 429 pM	5.2 ± 0.49 nM
25 (2001)	in solution (5′-fluoroph, 3′-quencher)	molecular beacon	change in fluoescence	TE (± Mg/K)	(2 mL) 5−40 nM TBA			10 nM
42 (2000)	5′-NH−C6 on silica beads optic fibers (microarray)	compet reusable	change in fluoescence (FITC-thrombin)	PBS	(7 μL) 100 nmol TBA 200 nM F- thrombin	15 min	1 nM	300 nM
39 (1998)	3′-C7 glass slide immobilized, 5′-FITC	molecular beacon reusable	evanescent wave-induced fluorescence anisotropy	PBS	(140 μL) 50 μM TBA	10 min	5 nM	47 nM

[a] The bibliographic reference, labeling or immobilization strategy, assay format, detection approach, binding buffer, concentration of biocomponents, assay time length, limit of detection, and dissociation constant reported by authors are reviewed.

aptamers not only avoids ethical problems but allows generation of aptamers against toxic or poorly immunogenic molecules, evades the limited genetic diversity characteristic of IgG and IgMs, and eludes the batch-to-batch variation distinctive of polyclonal Ab production. The combination of small-size aptamers toward different epitopes of a common target should admit the development of sandwich assays against molecules too small to be detected by sandwich ELISAs,[17,18] with the "capture" aptamer being selected against the target and the "reporter aptamer" against the aptamer−target complex.

Among their drawbacks, aptamers (as happens in general for free nucleic acids) are highly sensitive to nuclease attack, limiting their lifetime (and thus application). This problem has been successfully overcome in the shape of spiegelmers[19,20] and via the chemical modification of either the oligonucleotide backbone or the 2′ position of the pyrimidine moiety.[21,22] However, the production of a spiegelmer involves selection of a preliminary oligonucleotide against the synthetic enantiomer of the chosen target, which is not always feasible, and thus the route of chemical modification of the oligonucleotide library used in SELEX is more universally applicable.

The thrombin-binding aptamer (TBA, 5′-GGTTGGTGTGGTTGG-3′) was the first in vitro selected aptamer targeted toward a protein with no known physiological binding to nucleic acids,[23] and due to its potential applicability for anticlotting therapeutics, it has been extensively studied.[24−26] Under certain conditions, this oligomer is known to fold into a quadruplex structure, which contains two G(syn)−G(anti)−G(syn)−G(anti) quartets and three lateral loops, usually referred to as a "chair structure". Every group of four guanines array in a square planar configuration, each G interacting with the adjacent one via two hydrogen bonds and behaving as both H-bond acceptor and donor (a Hoogsteen base pair).[27−30] It has been described that the structure is stabilized by K^+ ions, and some authors defend that thrombin binding strongly depends on the presence of this ion. However, some published works have demonstrated that other cations (Pb^+, Ba^{2+}, Sr^{2+}) can bind and stabilize the quadruplex with higher efficiency than K^+,[24,31,32] while Li^+, Na^+, Cs^+, Mg^+, and Ca^{2+} form weaker complexes at low temperatures.[32,33] A recent report showed the selective binding of thrombin in the absence of ions.[34]

The first enzyme-linked oligonucleotide assay (ELONA) was described by Drolet in 1996[35] and consisted of a mixed ELISA/ELONA sandwich to detect human vascular endothelial growth factor on microtiter plates. Similar mixed assays have been set up on the surface of microbeads, using either immobilized or labeled aptamers as capture or detecting reagents.[36,37] However, the most interesting application of aptamers for analysis has been

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the demonstration of aptasensors[38−43] and aptamer molecular beacons.[25,26,44−47] Assays published by different authors making use of TBA to detect thrombin are summarized in Table 1. In most cases, it appears that methodology standardization has been strongly influenced by the authors' background, usually taking advantage of ELISA or nucleic acid-related protocols. Presumably for this reason, some reports have detailed the denaturation of aptamers to unfold any preexisting 3-D structures that may interfere with target recognition as is routine with DNA probes,[25,48] even though target recognition of aptamers depends on folding and 3-D structure rather than sequence. Furthermore, reports indicate the performance of incubations at 37 °C, as regularly carried out with antibodies, despite the fact that folding of most aptamers is favored at lower temperatures. To our knowledge, no reports have been made studying the importance of the different parameters playing a role in assay performance or comparing the efficiency of various ELONA formats. In this report, a systematic evaluation of the parameters with potential effect on ELONA performance has been carried out. Diverse ELONA formats exploiting the TBA were studied, and the effect of factors as pH, denaturation, presence of K^+, incubation time, and temperature were evaluated. ELONA results were confirmed using nuclear magnetic resonance (NMR), nondenaturing electrophoresis, and surface plasmon resonance (SPR). This study does not attempt to report that the conditions optimized for the TBA will be universal for all aptamers, but rather highlights that each aptamer will possess a unique structure for target binding that must be considered and assay conditions thus optimized on an individual aptamer basis.

MATERIAL AND METHODS

Biomolecules. HPLC purified and lyophilized oligonucleotides were provided by Cultek-Eurogentec (Belgium). Four variants of the biotinylated TBA with/without a six-carbon linker have been used: G12 (5′-biot-6C-GGTTGGTGTGGTTGGT), G13 (GGTTG-GTGTGGTTGGT-6C-biot-3′), G14 (5′-biot-GGTTGGTGTGGTTG-GT), and G15 (GGTTGGTGTGGTTGGT-Biot-3′). 5′-NH- and 3′-SH-modified TBA were used in the SPR experiments and for TBA bioconjugation. Human α-thrombin was supplied by Haematologic Technologies. Goat anti-human-thrombin Ab was purchased from Affinity Biologicals (Ancaster, Canada) and HRP-labeled anti-sheep IgG Ab was obtained from Sigma (Barcelona, Spain).

HRP (Sigma) was malemide activated using Sulfo-SMCC (Pierce) and conjugated to Ab/thrombin previously modified with SATA (Pierce).[49] Thrombin was biotinylated using *N*-hydroxysuccinimide-biotin (Sigma). The bioconjugates were desalted using G-25 columns (Amersham-Pharmacia, Barcelona, Spain), concentrated using Microcons (Millipore, Madrid, Spain) and quantified by UV−visible spectrophotometry. Enzyme activity was controlled before/after conjugation by diluting the conjugate in TMB/H_2O_2 (Sigma) and recording the increase in A_{650} nm for 5 min (ϵ 72 mM^{-1}; 1 enzyme unit oxidizes 0.1 nmol of TMB/min[50]).

General ELONA Protocol. Biotinylated biomolecules were immobilized on streptavidin-coated plates (SA plates; Thermo-Labsystems or NUNC) using PBS (50 mM PBS, pH 7.4) for 30 min at 37 °C. Nonbiotinylated molecules (e.g., thrombin, anti-thrombin antibody) were immobilized on NUNC Maxisorp plates using 50 mM carbonate buffer pH 9.6 for 1 h at 37 °C, followed by a 1-h blocking with PBS−Tween (10 mM pH 7.4, 0.05% v/v Tween 20). The plates were manually washed three times with PBS−Tween. If not specified, assays were performed in PBS, 50 mM KCl. Presence of the HRP enzyme label was detected using TMB and H_2O_2, and monitoring of color development was carried out at 650 nm, stopped after 20 min with 0.1 M H_2SO_4, and measured at 450 nm using a Wallac Victor^2 plate reader.

Effect of Buffer, pH, and [K^+]. A direct ELONA was exploited using the G13 TBA. G13 (50 nM in PBS) was immobilized for 30 min at 37 °C on SA plates. Following thorough washing, a 1:2 dilution series of 23 nM thrombin−HRP was applied across the plate to the immobilized TBA and incubated for 60 min at 4, 25, or 37 °C. Thrombin was diluted in either 10 mM PBS pH 7.4, 10 mM phosphate pH 7.4 or 50 mM Hepes buffer pH 8.0, containing 0−100 mM KCl, 0−50 mM NaCl, 0−3 mM MgCl, and 1% w/v casein or 0.05% v/v Tween as blocking agent in the experiments to study the effect of salt and buffer. The effect of pH was determined by carrying out the assay in 10 mM acetate pH 4.7, 10 mM PBS pH 6.5, 10 mM PBS pH 7.4, 10 mM PBS pH 8.5, or 10 mM carbonate pH 9.6, with 0.05% v/v Tween 20.

Effect of Incubation Time and Temperature. To study the effect of incubation time and temperature, the same direct ELONA was carried out, preparing a thrombin dilution series in PBS−Tween with 0−100 mM KCl. Incubations were carried at 4, 25, or 37 °C for 10−90 min.

Effect of Immobilization Orientation. To study the effect of aptamer immobilization, each of G12−G15 (50 mM in PBS) was immobilized on SA plates. The direct ELONA was carried out, performing 1-h incubations at 4, 25, and 37 °C in PB−Tween. Experiments were carried out in triplicate.

Effect of Predenaturation of TBA. To study the effect of aptamer denaturation on the ELONA performance, each of the TBA studied variants was diluted to a concentration of 50 nM in PBS and denatured by incubation for 10 min at 94 °C. Following rapid cooling, denatured/nondenatured TBA were immobilized on SA plates and the direct ELONA was performed.

Control Experiments. Various control experiments were carried out in parallel to each assay including the following: (i) use of a quadruplex-forming oligomer (5′- or 3′-biot-5′-GGGG-

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TTTTGGGG-3′,[51] (ii) lack of capture aptamer, (iii) nonspecific DNA, and (iv) using an alternative gliadin−HRP conjugate (chosen due to its similiarity in MW and p*I* to thrombin). The results of each assay are presented in terms of LOD (mean of blank ± 3 standard deviations), IC_{50} (thrombin concentration generating 50% of the maximum signal), and sensitivity (absorbance units per concentration (AU/nM). For the competition assay, these values were calculated adjusting the graphs to a second-order polynomic and using a quadratic equation solver. The concentration of reagents needed in each assay format was optimized by performing checkerboard titrations.[52]

NMR Experiments. ^{1}H NMR spectra were acquired at 500 MHz using a Varian Inova-500 NMR spectrometer. HPLC-purified and lyophilized TBA was diluted in 90% v/v H_2O/10% v/v D_2O to 2 mM. One-dimensional spectra were acquired at 4, 15, 25, and 37 °C before/after addition of KCl (final concentrations 10 and 50 mM).

Nondenaturing Gel Electrophoresis Experiments. Four-microliter samples were prepared containing 0.0657 pmol of thrombin (2.5 μg) and a 3:1, 1:1, or 1:3 molar ratio of TBA (0.197, 0.065, or 0.0219 pmol) or no TBA at all. All samples were prepared in 50 mM phosphate buffer pH 7.5 with or without 20 mM KCl and preincubated at room temperature for 20 min. Following incubation, 3 μL of loading buffer (30% v/v glycerol, 0.5% w/v bromophenol blue, in 60 mM Tris-HCl pH 6.8) was added to each sample. The samples were loaded in 6% w/v native acrylamide gels (37.1:1 acrylamide/bisacrylamide) prepared in 1× Tris−glycine buffer pH 8.3, with/without 20 mM KCl. Gels were run in the same 1× Tris−glycine buffer pH 8.3, with/without 20 mM KCl used for the gel casting, in a BioRad Microprotean III equipment at room temperature (10 V/cm). Staining was carried out by immersion of the gels in BioSafe Coommassie or silver staining (BioRad). Images of the gels were taken using a GenDoc image analyzer (BioRad).

ELISA/ELONA Experiments. In the capture format, HRP-labeled TBA and the dilution series of thrombin were preincubated in solution (PB−Tween, 50 mM KCl) for 30 min at 4 °C, to facilitate binding to each other, previous to capture on the Ab-coated wells for 60 min at 37 °C.

In the competition assays, immobilization of biotinylated TBA, biotinylated thrombin, or unmodified thrombin was carried as previously described. Following washing, a mixture of the competing molecules was added to each well. Competition was allowed to proceed for 15−90 min.

SPR Experiments. Immobilized Aptamer/Thrombin System. Thiol-modified TBA was immobilized on a bare gold chip (J1 Pioneer, BiaCore) by injecting 120 μL of 2 μM TBA in 1 M KH_2PO_4 buffer pH 3.5, with a flow of 2 μL/min at room temperature. The blocking step consisted of injecting 50 μL of 0.1 M mercaptoethanol in the same buffer, at a flow rate of 5 μL/min. Following washing, 10 μL of thrombin of 5, 11, 21, 60, 105, and 210 nM diluted in HBS-EP buffer (BiaCore) was injected at a flow rate of 2 μL/min. Association/dissociation constants were calculated using the BIAevaluation software. For the regeneration of the TBA-coated surface 50 mM glycine, 0.05% w/v SDS, and 2M NaCl were assayed.

Immobilized Thrombin/Labeled Aptamer System. Biotinylated thrombin was immobilized on streptavidin-coated chips (Sensor Chip SA Biacore). Due to the fact that no binding was observed, it was decided to prepare SA chips in-house using CM5 chips. Briefly, the surface of a CM5 chip was activated by addition of 30 μL of a 1:1 mixture of EDC (400 mM) and NHS (100 mM) at a flow rate of 5 μL/min. Twelve microliters of streptavidin was then added, followed by 30 μL of the blocking agent ethanolamine (1 M) and a final wash with 15 μL of HCl at a flow rate of 5 μL/min. Anti-thrombin Ab was immobilized on the surface of a CM5 chip in a similiar manner except that 30 μL of streptavidin was added instead of 12.

RESULTS

Determination of Parameters for Optimal ELONA Performance. Effect of Buffer and pH. Effect of pH was studied by performing a direct ELONA assay at 4 °C using incubation buffers of different pH with 50 mM KCl. Acetate buffer (50 mM pH 4.7), 10 mM PBS pH 6.5, 10 mM PBS pH 7.4, 10 mM PBS pH 8.5, and 50 mM carbonate−bicarbonate buffer pH 9.6 were assayed. To confirm that the effect of pH on assay performance was not attributable to loss of activity of the thrombin enzyme label, its activity was measured pre- and postincubation. No notable loss of activity was observed, indicating that any effect noted would be due to the influence of pH on TBA or thrombin stability or binding ability. The direct ELONA gave similar results in the pH range 4.7−8.5. Lower signals and higher backgrounds were obtained under more extreme pHs, probably due to denaturation of some of the biocomponents. Phosphate buffer (50 mM pH 7.5) and 10 mM HEPES pH 8.0 were successfully used in place of PBS, and better thrombin binding was detected in absence of KCl when HEPES was used.

Effect of [K^+]. The dependence of the quadruplex formation on the presence of certain cations has been discussed extensively. Several authors have, for example, demonstrated that different ions may have a stabilizing effect on certain quadruplexes, and they may induce structural transitions measurable in terms of changes in quartet diameter,[53] type of quadruplex,[30,54] loop alignment,[24,55] or changes in melting temperature[33] or absorbance pattern.[24,33] Miyoshi et al.[54] suggested that not only ionic radius but also other properties of cations, including the energy of dehydration or coordination number, must be considered in order to elucidate the general rules of the effect of cations on quadruplex structures. Additionally, it has been demonstrated that different ions may have different number and location of binding sites within the quadruplex.[24,30,56]

Specifically, the TBA is known to be in equilibrium between a random and a quadruplex conformation when dissolved in aqueous solution at room temperature (Figure 1).[26] K^+ is believed to shift this equilibrium toward the quadruplex conformation, subsequently favoring thrombin binding,[23,27−29] and it has been reported that the quadruplex is not detectable without K^+ in the

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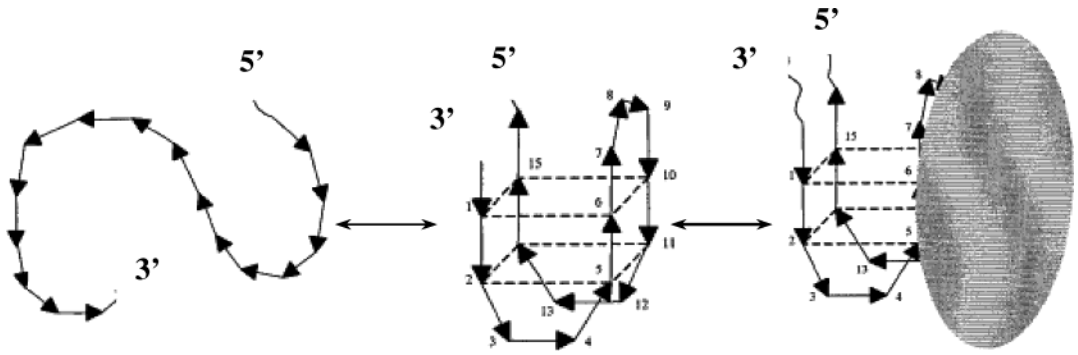

Figure 1. TBA equilibrium between random (left) and quadruplex (right) conformations when diluted in an aqueous solution. In the latter one, H-bonds are established between guanines. It is believed that K^+ shifts the equilibrium toward the quadruplex folding.

medium.[24,57] On the other hand, reports indicate that the presence of K^+ is more important when approaching the TBA melting temperature (T_m) and thus thermodynamic instability of the quadruplex is expected. To this end, experiments were carried out in the absence and presence of different concentrations of KCl, at different temperatures.

The presence or absence of KCl appeared not to play an important role at lower temperatures (4 and 25 °C), but when temperature was further increased (37 and 45 °C), a notable difference in achievable detection limits was observed in the presence (10−50 mM) and absence of KCl (Figure 2). No thrombin binding is detected when incubation is carried out at 65 °C. These results appear to indicate that (i) the quadruplex structure of the TBA is thermodynamically stable at lower temperatures, even in the absence of KCl. (ii) As the TBA is bound to the thrombin target, the equilibrium continuously shifts to the formation of quadruplex from random coil. (iii) As the temperature approaches the T_m of the TBA, it becomes thermodynamically instable and the presence of K^+ facilitates the maintenance of structural integrity. Increasing the concentration of K^+ (100 mM) did not result in improved assay performance. In our experiments, addition of Na^+ or Mg^{2+} to K^+ consistently resulted in lowered performance compared to KCl alone. We have observed that immobilization via the 5′-terminal of the TBA diminishes the ability for folding. It was observed that the dependence of thrombin binding (and quadruplex formation) on the presence of K^+ was more emphasized in the case of the 5′-immobilized TBA. It can be concluded that the presence of K^+ stabilizes quadruplex formation when nonoptimal conditions for aptamer folding are used (high temperature, nonfavorable immobilization, etc), but that under optimal conditions (low temperature, favorable immobilization), the presence of K^+ is not critical.

Effect of Incubation Temperature. The study of the effect of incubation temperature was achieved by carrying out the direct ELONA at different temperatures. A dilution series of HRP−thrombin was incubated for 1 h on immobilized G13−TBA at 4, 24, 37, 45, and 65 °C in PB−Tween, 0−100 mM KCl. The highest sensitivity and maximum values were obtained at 4 °C, independently of the incubation buffer or the KCl content (data not shown). The result confirmed that the quadruplex structure (i) is stable under low temperature even in the absence of stabilizing ions and (ii) is promoted/stabilized by thrombin binding under these conditions. The assay efficiency decreased as temperature increased, and such an effect was more evident when the incubation buffer lacked KCl. This is coherent with the evidence that K^+ cations stabilize the TBA quadruplex and increase its T_m. Consequently, very low thrombin binding and no binding at all could be detected after incubation at 45 and 65 °C, respectively, either in the presence or in the absence of KCl. This confirms that thrombin does not bind the TBA when the temperature of incubation approaches or surpasses its T_m, and thus TBA cannot maintain its folded structure.

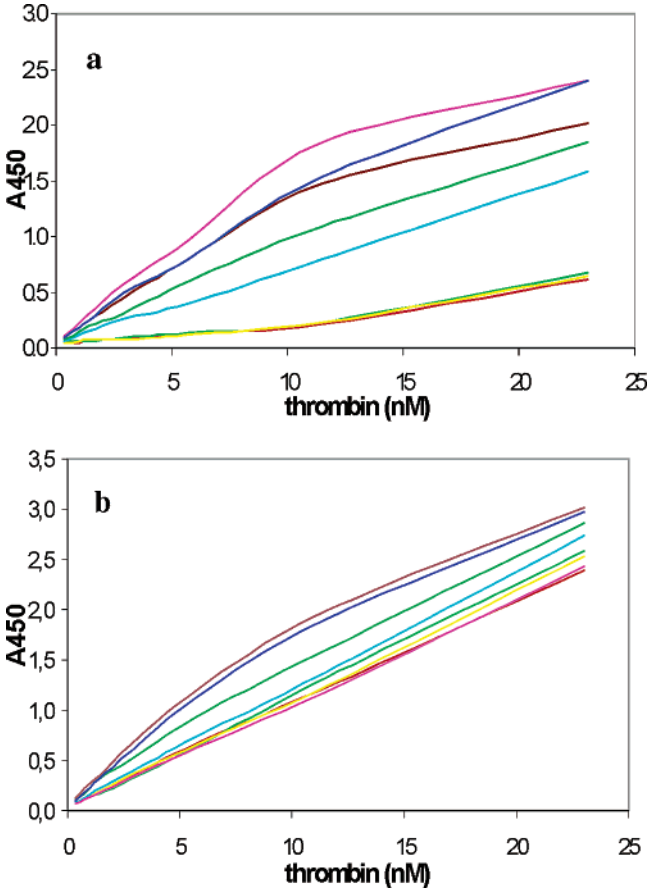

Figure 2. Effect of K^+ on the ELONA performance at 37 (a) or 4 °C (b). The TBA was immobilized on microtitration plates and used to detect a dilution series of HRP-thrombin. Incubation buffer: (red) 10 mM phosphate buffer alone or with (green) 5 μM KCl; (yellow) 5 μM KCl, 50 mM NaCl, 3 mM MgCl; (blue) 10 mM KCl; (green) 10 mM KCl, 50 mM NaCl, 3 mM MgCl; (red) 50 mM KCl; (light purple) 50 mM KCl, 50 mM NaCl, 3 mM MgCl; (dark purple) 100 mM KCl.

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Table 2. Performance of the Four TBA Variants under Study, Depending on the Immobilization Strategy[a]

TBA variant	IC_{50} (nM) a/b	sensitivity (AU/nM) a/b	LOD (nM) a/b
G12	10/13.6	$7.2 \times 10^{-3}/6.9 \times 10^{-3}$	4/3.8
G13	10/15.1	$5.2 \times 10^{-2}/6.6 \times 10^{-2}$	2/1.8
G14	40/32.6	$4.4 \times 10^{-3}/3.2 \times 10^{-3}$	12/6.0
G15	25/25.6	$3.4 \times 10^{-3}/1.9 \times 10^{-3}$	10/9.0

[a] Following immobilization of each of four TBA variants on streptavidin-coated plates, a direct (a) or a competition ELONA (b) was carried out and the thrombin binding efficiency of each variant was evaluated in terms of IC_{50}, sensitivity, and LOD (defined as the mean of the blanks ± 3 standard deviations).

Effect of Incubation Time. The study of the effect of the incubation time on binding efficiency was carried out for both the direct and competition ELONA formats. G13 was immobilized on streptavidin-coated plates, and capture of (i) HRP−thrombin or (ii) competing labeled/unlabeled thrombin was allowed to occur for 10, 20, 30, 45, 60, or 90 min. As expected, longer incubation times generated increased binding efficiencies in the direct ELONA format. As the aim was to optimize an assay to be both as quick and as efficient as possible, it was decided to perform 1-h incubations in further experiments. In the competition format, improvement of binding was observed while incubation time increased from 10 to 30 min. Inversely, incubations longer than 30 min correlated with a decline in binding efficiency. The reason was demonstrated to be that labeled thrombin behaves as a suboptimal target of the TBA compared to the nonlabeled thrombin. The former is thus displaced by the latter during long periods of incubation. Work is ongoing to develop a displacement assay exploiting the oberved behavior.

Effect of Immobilization Orientation. Most authors have used the TBA to detect thrombin in solution,[25,26,34] and only two reports make use of immobilized TBA[39,42] (Table 1). Given the requirement of aptamers to adopt a certain structure to facilitate target binding, a study was carried out to probe the effect of immobilization on the ability of the TBA to fold into the quadruplex structure required for binding of thrombin. Thus, four variants of the TBA were chosen in order to facilitate immobilization via either its 3′- or 5′-terminal; an additional 6C-linker was included or not to study whether this spacer arm allowed increased flexibility of the TBA to form the 3-D quadruplex structure. A direct ELONA was carried out by adding a dilution series of thrombin−HRP to each of the immobilized TBA variants. As indicated in Table 2, immobilization through the TBA 3′-end improved binding of thrombin−HRP compared to immobilization through the 5′. These results suggest that either 5′-modification/immobilization interferes with the correct folding of this aptamer, and thus its ability to bind thrombin, or, alternatively, that 3′-modification improves structural stability. As expected, addition of a linker correlated with improved target binding in both cases, presumably due to the increased spatial differentiation between the TBA and the surface while decreasing steric hindrance, resulting in improved folding and thrombin recognition.

These conclusions were confirmed by the results of competition assays performed with the same four TBA variants. As can be seen in Table 2, the best performance in terms of lowest LOD and IC_{50} (2 and 10 nM) and highest sensitivity (5.2×10^{-5} AU/nM) was obtained using G13. The LOD increased to 10−12 nM and the IC_{50} to 40−25 nM when the spacer arm was not employed for 5′- and 3′-immobilization, respectively. Interestingly, it has been previously described that addition of one guanine to the 5′-end of TBA correlates with increased instability in solution, while the same addition to the 3′ improves stability,[24] agreeing with our observations.

Effect of Aptamer Denaturation. The TBA was denatured by incubation for 10 min at 94 °C, followed by rapid cooling on ice. This methodology is routinely executed with DNA probes with the aim of defolding any preexisting 3-D structures that may interfere with target binding.[25,48] Denatured or nondenatured G13−TBA was immobilized on SA plates and used in the previously described direct ELONA in order to compare their thrombin binding efficiency. According to our results, aptamer denaturation did not improve efficiency of the assays performed at either 4 or 37 °C. This may indicate that TBA adopts the same folding before/after denaturation under the studied conditions or that the presence of thrombin promotes a certain folding. This is coherent with the fact that the TBA folding has been described to follow very fast kinetics, especially when the buffer contains ≥10 mM KCl.[58] On the other hand, Marathias and Bolton[30] reported that some quadruplex-forming DNAs produce structure mixtures when heated to 80 °C and slowly cooled but a single conformation after rapid cooling. The TBA seems to produce just a single conformation in both cases. In conclusion, denaturation of the aptamer may not improve its binding efficiency when (i) the structure responsible for target recognition already predominates in the solution or (ii) target presence or binding promotes folding of a certain structure. However, aptamer melting may be necessary if the oligomer is known to adopt very stable conformations susceptible to compete/kidnap a minoritary or less stable structure needed for target binding (not the case for the TBA, but may be the case for other aptamers).

Control Experiments. The possibility that the observed thrombin binding could be due to nonspecific binding was ruled out by systematically adding a series of negative controls to each of the performed assays. Immobilization of another quadruplex-forming oligomer (5′-biotin-GGGGTTTTGGGG-3′ or 5′-GGGGTT-TTGGGG-biotin-3′[51]) or a DNA not described to form quadruplexes always correlated to lack of binding and generated lower backgrounds that the controls lacking any coating (probably blocking the surface or providing it with negative charge). On the other hand, addition of another protein labeled with HRP did not produce significant binding on the immobilized TBA.

Characterization of the TBA Folding in the Presence/Absence of KCl and Thrombin. H^+ NMR Study. It is known that the TBA quadruplex structure formed in the presence of KCl is stabilized by eight H-bonds produced between the four guanines of each G-quartet.[28] These imino protons can be detected by H^+ NMR as chemical shifts in the range 10.5−12 ppm. To study the structure of TBA in the absence of both cations and thrombin, a series of H^+ NMR spectra was obtained while increasing the temperature (4, 15, 25, and 37 °C) of TBA dissolved in ultrapure water. Subsequently, potassium was added (final concentration 10 and 50 mM) and the experiment was repeated (Figure 3). The spectrum obtained in the presence of 10 mM KCl was as

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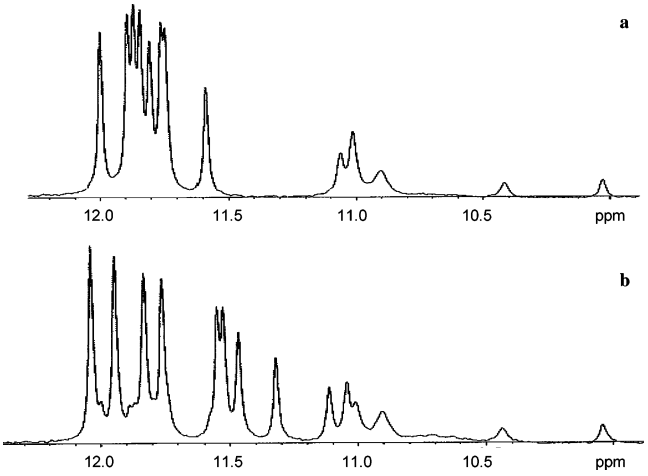

Figure 3. Effect of KCl and temperature on the NMR spectra. ^{1}H NMR spectra were acquired at 500 MHz from HPLC-purified and lyophiliZed TBA diluted in 90:10% v/v H_2O/D_2O to 2 mM. The one-dimensional spectra shown were acquired at FOUR in the absence (a) or in the presence of KCl (b).

previously reported.[27,28] Increasing the KCl concentration did not induce detectable changes at any of the tested temperatures. It could be observed that, at 4 °C and without K^+ in the medium, eight imino proton chemical shifts resonate in the region characteristic of guanine imino protons involved in GGGG tetrad formation. It was also observed that four of the imine protons appear displaced downfield compared to spectra obtained in the presence of K^+. Our results seem to indicate that a quadruplex structure is formed in the absence of K^+, but its intramolecular configuration is slightly different from the one associated to the presence of K^+ ions. Addition of Pb^+ or Mn^+ also seem to induce changes in the TBA NMR pattern,[24,30] and a largely unresolved spectrum of the nonexchangeable protons in all regions at 15 °C has previously been reported in the presence of Na^+.[30] These ions are known to induce formation of a quadruplex structure slightly different from the one published for K^+. Kankia and Marky[33] for example, observed that the quadruplex complex formed in the presence of Sr^+ is tighter than the one formed with K^+ due to the higher charge of the Sr^+ ion.

The effect of increase in temperature was evaluated. In these experiments, temperature increase had almost no effect on the shifts related to tetrad formation when salt was present. It has been previously described that K^+ increases TBA T_m by stabilizing its folded structure.[33] In the absence of KCl, the spectrum in this range was conserved until the temperature reached 15 °C. Above this temperature not only the height of the peaks decrease, but the overall spectrum seems to differ slightly. In conclusion, a higher destabilizing effect could be related to temperature increase when no salt was present in the medium, reflecting ELONA results obtained. Change in structure due to addition of KCl was immediately observed. Jing and Hogan[58] also described very fast kinetics of the TBA quadruplex formation when they measured changes in UV absorvance at 264 nM in the 0−300-s time range, after addition of KCl (0.02−1 mM).

Electrophoresis Study. The thrombin−TBA interaction was also studied using electrophoresis. Thrombin was incubated for 20 min at room temperature in the presence of different molar ratios of the TBA (3:1, 1:1, 1:3), and run in a nondenaturing acrylamide gel. The incubations were performed in 50 mM phosphate buffer pH 7.5 both in the presence and in the absence of K^+. Thrombin alone was preincubated in the same conditions as a negative control. As can be observed in Figure 4, preincubation of thrombin with equivalent or higher molar ratios of the TBA induced improved migration of the protein in all the performed experiments. Preincubation of thrombin with a lower amount of TBA generates a major band in the gel, which shows as low mobility as thrombin alone and traces of a second band with increased mobility. Similar results were obtained in the presence and in the absence of KCl. Thrombin is know to have a p*I* of 7−7.5 and is thus expected to remain almost neutral in the studied electrophoretic conditions and exhibit quite a limited mobility. The results indicate that the TBA binds thrombin even in the absence of KCl and consequently provides it with added electrical charge, demonstrated by its electrophoretic mobility.

ELONA and Mixed ELISA/ELONA Development. The efficiency of different assay formats (Figure 5) was compared and outlined in Table 3.

Mixed Antibody/Aptamer Sandwich Format. Mixed Ab/aptamer sandwich formats have been successfully performed by immobilizing TBA and detecting the aptamer-captured thrombin with labeled antibodies. In the indirect sandwich format, a sheep anti-thrombin Ab was used to bind the captured thrombin and was detected by an HRP-labeled anti-sheep Ab (Figure 5a). For the direct format, the anti-thrombin Ab was chemically conjugated to HRP. The LOD of both assays, independently of the TBA variant used, was <1 nM, and the IC_{50} was 2 nM. The indirect format

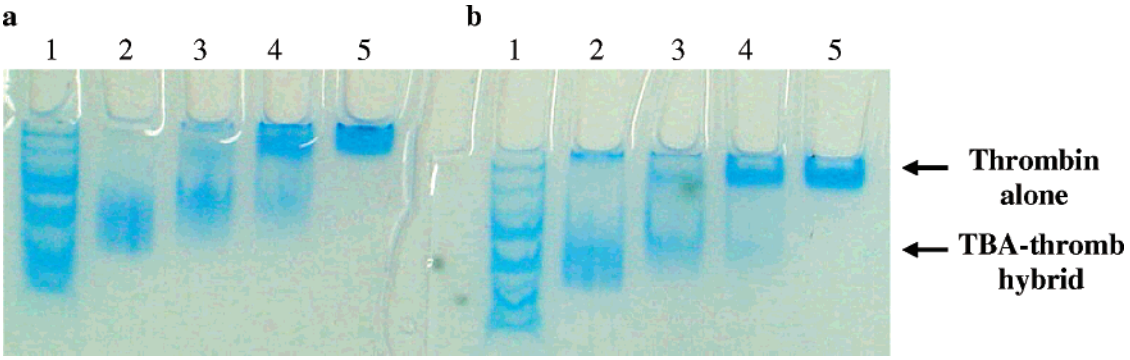

Figure 4. Nondenaturing electrophoresis of thrombin (2.5 μg) following incubation with different molar ratios of TBA or with no TBA at all. The experiment was carried out in either the absence (a) or the presence (b) of 20 mM KCl. Rows 1−5 indicate the following: 1, protein ladder; 2, 3:1 TBA/thrombin molar ratio; 3, 1:1 TBA/thrombin molar ratio; 4, 1:3 TBA/thrombin molar ratio; 5, thrombin incubated alone.

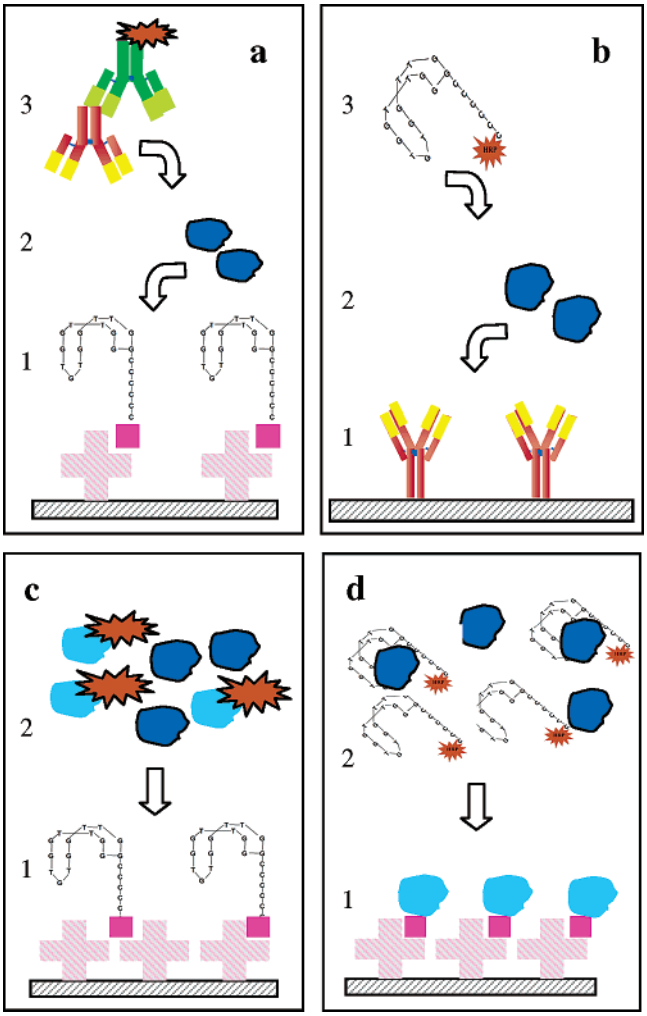

Figure 5. Assayed ELONA and mixed ELISA/ELONA formats. Biotinylated TBA was immobilized on streptavidin-coated microtiter plates (a, c, d). For Ab immobilization, Maxisorp NUNC plates were used (b). (a) Indirect mixed sandwich: thrombin captured on immobilized TBA is bound by anti-thrombin sheep-Ab and detected with labeled anti-sheep Ab. The direct mixed sandwich is similar except that HRP-conjugated anti-thrombin Ab is used. (b) Inverse sandwich format: thrombin captured by immobilized anti-thrombin Ab is detected by labeled TBA. A capture inverse sandwich was also attempted by preincubating thrombin and HRP−TBA in solution before being captured by immobilized anti-thrombin Ab. (c) Competitive ELONA: labeled and nonlabeled thrombin in solution compete for the immobilized TBA. (d) Inverse competition: unmodified thrombin in solution and immobilized thrombin compete for labeled TBA in solution.

showed to be a little bit more sensitive, presumably due to (a) the nonaffected affinity of the unmodified anti-thrombin Ab or (b) to differences in the activities of the HRP linked to the labeled Abs. The indirect sandwich was successfully shortened by preincubating the two sets of Abs for 1 h.

An inverse format was attempted by immobilizing the anti-thrombin Ab and detecting thrombin using enzyme-labeled TBA (Figure 5b). This assay appeared to have very high LOD (20−100 nM) and background, very low maximal values, and at the same time, it required notably elevated concentrations of all the reagents used, thus significantly increasing the assay cost. The inverse assay gave reasonable results only when performed as a capture assay. For this approach, thrombin and labeled TBA were preincubated in solution before being added to the immobilized

Table 3. Summary of the Results Obtained for the ELONA and Mixed ELISA/ELONA Formats Reported in This Work

assay format	reagent concn	LOD (nM)	IC_{50} (nM)	sensitivity (AU/nM)	assay time (h)
direct mixed sandwich	50 nM biotin−TBA	<1	2	0.0045	3
indirect mixed sandwich	50 nM biotin−TBA	<1	2	0.008	3−4
inverse mixed sandwich		>20	50		4
capture inverse mixed sandwich					2
+ KCl		<1.5	3.5	0.0043	
− Cl		<1	<2	0.0038	
competition	50 nM biotin−TBA 17 nM HRP−thrombin	1.8	15	0.0066	1.5
inversed competition	6.4 μM HRP−TBA 660 nM immobilized thrombin				>3
ELONA double sandwich	50 nM biotin−TBA 350 nM HRP−TBA				2.5

anti-thrombin Ab. This assay had IC_{50} of 3.5 nM and LOD < 1.5 nM when performed in the presence of KCl, and IC_{50} < 2 nM and LOD < 1 nM in the absence of KCl. These results appear to indicate that it is necessary to have thrombin in a free uncomplexed form for the TBA to correctly recognize and fold around it. Moreover, the concentrations of bioconjugates required were high and the maximal signals obtained were 3−4 times lower than in the direct assays. This effect was believed to be due to steric hindrance caused by conjugation of enzyme to the smaller aptamer. To confirm this, a control experiment was carried out by immobilizing anti-thrombin Ab and adding (i) preincubated thrombin−G13 mixture or (ii) thrombin followed by G13. Streptavidin−HRP was then added. In both cases, results similar to the immobilized aptamer sandwich format were obtained, supporting the belief that the poor results obtained are not due to the hindrance of access to thrombin sites for aptamer binding, due to being bound to antibody, but rather due to the TBA−HRP. Aptamer folding appears to be hampered when the aptamer is conjugated to large molecules such as HRP but not affected when conjugated to small molecules such as biotin. Work is ongoing to probe whether the inhibition of aptamer structure formation by enzyme labels is a problem unique to the TBA.

ELONA Competition. A competition assay, based on TBA immobilization followed by competition between labeled and nonlabeled thrombin, was successfully optimized (Figure 5c). This assay shows LOD of 1.8−9 nM and IC_{50} of 13−32.5 nM, depending on the TBA variant in use (Table 2). The optimum results were obtained when coating the TBA through its 3′-end with a 6-C linker, confirming that 5′-immobilization somehow interferes with the correct folding of the TBA and, thus, its ability to bind thrombin. Consistently, addition of a linker improves folding, probably due to a decrease in steric hindrance.

The inverse format (coat thrombin or biotinylated thrombin, followed by competition between immobilized and free thrombin for HRP−TBA in solution) was also studied (Figure 5d). Preliminary checkerboard results seemed to confirm that the TBA 3′-modification worked well, while modifying the 5′-end had a detrimental effect on the assay performance. However, when a competition was performed, “sandwich” like results were obtained. This indicated that, apart from the labeled TBA binding the

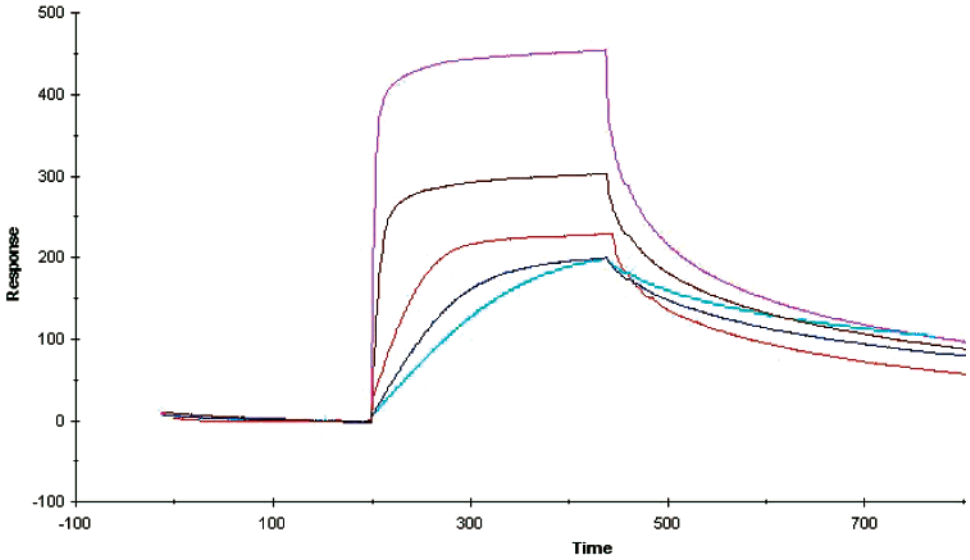

Figure 6. SPR evaluation of immobilized aptamer/thrombin interaction. Sensorgrams of thiol-modified TBA immobilized on J1 pioneer gold chip (Biacore) and sequential additions of thrombin (5, 11, 21, 60, 105, and 210 nM). The flow rate was 2 μL/min, and the regeneration agent employed was 2 M NaCl. The BIAEVAL software was used to calculate the K_D, which was determined to be 6.61 $\times$ 10^{-9} M.

immobilized thrombin, the complex formed by TBA−thrombin in solution was also trapped on the plate surface. The alternative capture of biotinylated thrombin on SA plates did not improve the result or decrease background. Consistent with previous results, this format requires very high concentrations of all reagents compared to the opposite configuration (6.4 μM G13−HRP versus 17 nM thrombin−HRP, and 660 nM immobilized thrombin versus 50 nM G13). To test whether immobilization of thrombin resulted in modification of its structure or masking of the TBA target sequence, an experiment was carried out using immobilized thrombin and labeled anti-thrombin Ab. Thrombin was clearly detected with LOD < 10 nM. This further supports results obtained with the inverse sandwich assay where enhanced binding was obtained after preincubating TBA with thrombin in solution, indicating that for effective detection of the target the aptamer requires the flexibility to "fold around" the thrombin.

ELONA Double Sandwich. The analysis of the TBA crystal structure published by Padmanabhan et al.[29] indicated that the TBA was sandwiched between two symmetrically related thrombin molecules. The model obtained using NMR placed the TT loops in proximity to the fibrinogen recognition site of one thrombin molecule (exosite 1) and the TGT loop close to the heparin binding site of a second thrombin molecule (exosite 2).[60] Contrary to that, directed mutagenesis studies performed by Tsiang et al.[61] indicated that either the TBA does not bind exosite 2 or that exosite 2 binding is nonihibitory. These authors proposed that interactions to this site may be unique to the crystal structure.

An ELONA double sandwich was attempted by immobilizing G13, capturing different concentrations of thrombin, and detecting it with HPR-labeled TBA. A certain trend was observed, but the maximal values, LOD, and sensitivity were very low. Consistent with previous results, high concentrations of the labeled aptamer were needed (350 nM compared to 8−25 nM HRP−thrombin used in the competition format). This again agrees with the observation that effective recognition of thrombin requires that the TBA is free and in a noncomplexed form. Although aptamers were not available to study the true ability of double aptamer sandwich assays, where the capture aptamer is selected against the target and the reporter aptamer is selected against the aptamer−target complex, it is predicted that this type of assay may offer considerable advantages over their antibody homologues.

SPR Study. Immobilized Aptamer/Thrombin System. Thiolated TBA was immobilized on a gold chip, the surface was blocked, and different concentrations of thrombin were added (5, 11, 21, 60, 105, and 210 nM; Figure 6). Higher concentrations saturated the sensor, and no further signal increase was detected. Three different solutions were assayed to regenerate the TBA-coated surface before each new thrombin addition: 50 mM glycine, 0.05% w/v SDS, and 2 M NaCl. The best regenerating agent appeared to be 2 M NaCl. Presumably this solution induces a change in TBA or thrombin folding, thus affecting their binding, without damaging the oligomer structure and allowing recovery of the quadruplex as soon as the regeneration solution is replaced by the binding one.

In agreement with ELISA/ELONA results, immobilization via the 5′-moiety resulted in decreased binding efficiency and higher dissociation constants. Additionally, the incorporation of the spacer molecule was also observed to have a beneficial effect on binding efficiency. The best K_D value was obtained with the G13−TBA, being determined to be 6.6 $\times$ 10^{-9} M.

Immobilized Thrombin/Labeled Aptamer System. Inversely, biotinylated thrombin was immobilized on a streptavidin-coated chip. Initially this was attempted using a Biacore SA chip. However, no binding of biotinylated thrombin was observed (ΔRU = 1.2), and thus, a CM5 chip was functionalized with a layer of streptavidin and biotinylated thrombin observed to bind very efficiently (ΔRU = 523). Upon addition of HRP-labeled aptamer to the chip, no interaction with the immobilized thrombin was noted. This could be attributed to degradation of the biotinylated

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(60) Kelly, J. A.; Feigon, J.; Yeates, T. O. *J. Mol. Biol.* **1996**, *256*, 417−22.
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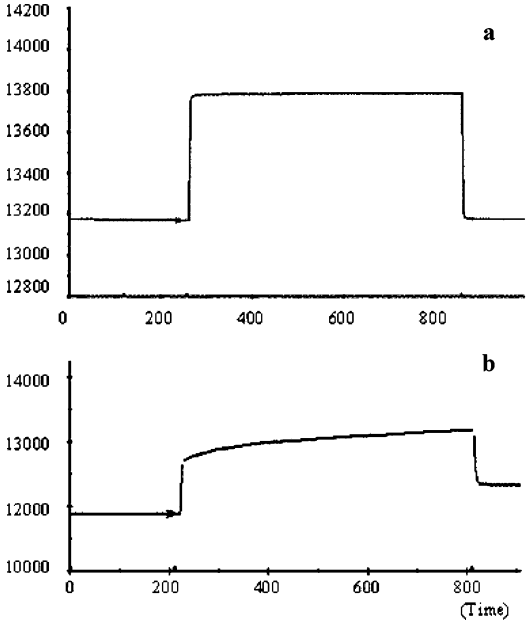

Figure 7. SPR evaluation of immobilized thrombin/labeled aptamer interaction. Monitoring of immobilized thrombin/labeled aptamer interaction using Biacore 1000. Thrombin immobilized via EDC/NHS on CM5 chip (Biacore) and blocked with ethanolamine. Following washing, (a) HRP-labeled aptamer (b) anti-thrombin antibody was added at a flow rate of 2 μL/min, and the regeneration agent employed was 2 M NaCl.

thrombin or to loss of sensitivity due to the streptavidin layer increasing distance from the chip surface. Thus, thrombin was immobilized directly onto a CM5 chip via carbodiimide chemistry, the HRP−TBA was added to the surface, but again no interaction was observed. To probe whether thrombin immobilization on the CM5 chip resulted in degradation or steric hindrance, anti-thrombin Ab was added. Very strong interaction was observed (Figure 7) thus indicating, as observed in previous ELISA/ELONA experiments, that once the aptamer is labeled with a large molecule, such as an enzyme, its ability to fold into the quadruplex structure necessary for binding is severely hampered. Work is ongoing to see if this is also the case with other aptamers or if this property is unique to the TBA.

DISCUSSION

In the reported work, a direct ELONA assay has been optimized and applied to the study of the potential influence of several external factors in the thrombin−TBA binding. It has been concluded that denaturation of TBA is not needed for the assay's optimal performance and that a wide buffer pH range is tolerated. However, other factors appear to affect assay efficiency. For example, incubation time had to be optimized for each assay format, and aptamer immobilization decreased its binding efficiency if a linker was not included.

In all the assayed formats, the best thrombin binding has been observed when TBA has been immobilized through its 3′-extreme instead of the 5′. Other authors have successfully used 5′-immobilized TBA on optic fibers, using a 6-C linker.[42] Although the authors report high sensitivity (LOD = 10 nM thrombin for the direct format and 1 nM for the competition one), the calculated K_d (300 nM) is higher than the previously published data. On the other hand, increased instability of TBA in solution has been previously described after addition of one guanine to its 5′. The same modification to the 3′ showed improved stability.[24] Similarly, Dougan et al.[62] observed that 3′-biotinylated TBA displayed extended lifetimes when incubated in blood samples. This was related to the existence of serum 3′-exonucleases as, in fact, 3′-end capping was enough to extend lifetime of this aptamer in serum. In the present study, extended incubations of the direct assay (up to 90 min) always related to increased binding efficiency, suggesting that extended degradation of the TBA was not taking place. At the same time, addition of a 6-C linker proportionally reduced differences between both immobilizations in terms of LOD and sensitivity. We suggest that 5′-immobilization may be somehow interfering or affecting the TBA folding process and, thus, thrombin recognition. Other authors have detected weakened thrombin inhibitory activity of 5′-biotinylated TBA compared to the unmodified.[63]

The potential effect of K^+ on TBA−thrombin recognition has been assayed. Binding in the absence of K^+ has been shown to occur and to be as efficient as in the presence of K^+ at low temperature (4−24 °C). As bound complexes have only been observed in the quadruplex form of TBA, this implies that either the aptamer folds in a certain structure that is stable at low temperatures, well below the TBA T_m, or that thrombin can somehow stabilize and perhaps even promote the quadruplex formation. As temperature approaches T_m, the importance of salt as a structure-stabilizing agent increases. Coherently, the TBA T_m has been described to be higher in the presence of KCl than in its absence[33] and dependent on the ionic strength, which results in a T_m shift to higher values as the KCl concentration increases (46.7 °C in 10 mM KCl, and 55.2 °C in 250 mM KCl). At the same time, T_m is known to be different in the presence of different ions: Sr^+ (63.1 °C) > Ba^{2+} > K^+ (48.7 °C) ≫ Rb^+ ∼ NH_4^+ (29 °C) and Cs^+ (15 °C).[64]

A decrease in binding efficiency has been observed following addition of Na^+ or Mg^{2+} to the KCl-containing buffer, which is consistent with published data indicating that the folding of DNA G-quartets is affected by these ions. As an example, Keniry[53] published that Na^+ and K^+ interact at different points of the TBA quadruplex, mainly due to their difference in size, inducing different diameters to the G-quartets. Slightly different quadruplex structures may show different affinities for thrombin.

The effect of the presence or absence of K^+ was confirmed by electrophoresis and NMR. An H^+ NMR spectrum, consisting of eight chemical shifts in the range characteristic of G-quartet formation, was obtained at low temperature (4−25 °C) in the absence of KCl. These results suggest that the TBA can fold into a quadruplex structure under these conditions, even though such a structure is slightly different from the one reported in the presence of K^+. As the TBA quadruplex is known to be tightened by certain ions in the medium, and presumably favored/stabilized by thrombin, it is consistent if in the absence of these factors the TBA folds into a weaker quadruplex, with the folded structure shown to be more resistant to the increase in temperature in the presence of KCl.

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The reported results may suggest that thrombin binding to the quadruplex-folded TBA stabilizes this conformation, or even promotes its formation, thus shifting the equilibrium between random and quadruplex form toward the latter one. The first assumption is highly consistent with the fact that this interaction is extremely stable. The correct functioning of a TBA molecular beacon whose folding was promoted by thrombin under low KCl concentrations at room temperature supports such an assumption.[25,26] Additionally, Ho and Leclerc[34] reported similar results when studying TBA complexed to K^+ or with thrombin, following addition of a water-soluble cationic polythiophene, which develops different colors when interacted with unfolded/folded DNA. These results indicate that TBA assumes a similar folded structure under both situations, and as this structure-related color is not observed in the absence of both thrombin and K^+, the authors concluded that thrombin promotes TBA quadruplex formation, even in the absence of K^+. Thus, we propose that addition of *either* KCl or thrombin may tighten the structure in agreement with reports that proteins promote quadruplex formation of telomeric sequences.[63,65] These proteins seem to enhance the rate of already thermodynamically favored transitions, acting as molecular chaperones. A possible mechanism is that they act as a surface on which DNA strands are brought together, effectively increasing local concentration and allowing quadruplex formation at relatively low concentrations.

Diverse ELONA and mixed ELISA/ELONA formats have been studied. Sandwich assays involving immobilized TBA and labeled antibody facilitated the lowest detection limits, with no differences being observed in assay performance when the TBA was immobilized through either the 5′- or 3′-moieties or in the presence/absence of a spacer. The inverse format involving immobilized Ab and labeled aptamer demonstrated a notable loss in sensitivity, increased detection limit, and reagent consumption. Replacing the enzyme label in this assay with biotin and detecting with SA−HRP allowed complete recovery of sensitivity and detectability. This observation demonstrates that the loss in assay performance is not attributable to a lack of ability of the TBA to fold around the thrombin once complexed with antibody, but rather that the labeling of the TBA with large enzyme molecules inhibits its ability to fold into the quadruplex necessary for thrombin binding.

Competition formats were demonstrated to be sensitive, rapid assays with detection limits of <10 nM. Interestingly, the effect of immobilizing the TBA via the 5′-/3′-moieties and the presence of a spacer molecule had a pronounced effect on this format performance.

CONCLUSION

Various formats of sandwich and competition assays exploiting the thrombin binding aptamer have been developed. Sandwich assays were the most sensitive but also the most time-consuming. The most interesting assay was the competition, which coupled sensitivity and assay time. A systematic evaluation of the parameters with potential effect on the ELONA performance has been carried out. Labeling of the TBA was observed to seriously inhibit its ability to fold into the quadruplex structure required for thrombin binding. Immobilization of the TBA through the 5′-/3′-extreemity and the presence of a spacer molecule were observed to affect assay performance of the competition assay but did not affect sandwich assay results. SPR studies carried out using Biacore 1000 confirmed these results. A study of the effect of pH and buffer concluded that the assay can be carried out in a wide pH range (4.7−8.5). Incubation temperature had a marked effect on assay performance with better results obtained at lower temperatures, optimally at 4 °C. Increasing the incubation time of the competition assay was observed to induce a displacement-type effect, presumably due to equilibrium shifts. Predenaturation of the TBA to unfold its 3-D structure prior to assay was not required and in fact was detrimental to assay performance. The presence of K^+ was not observed to be necessary at lower temperatures observed to be optimal for thrombin binding. However, when the temperature is increased toward the T_m of the TBA, the presence of K^+ has a pronounced effect, with this effect being more strongly observed with TBA immobilized through the 5′-terminal. Thus, we postulate that K^+ is not necessary for quadruplex formation but rather stabilizes the three-dimensional structure, particularly under nonoptimal conditions (elevated temperature/unfavorable immobilization). NMR and electrophoresis studies corroborated these results, and it can be believed that thrombin itself encourages the quadruplex formation, continuously shifting the equilibrium between random coil and quadruplex conformations toward the latter, as the thrombin is bound.

The use of aptamers represents new and exciting possibilities for analytical assays and devices with reagentless, washless formats facile to achieve. As suggested in this work, each aptamer is unique to its structure and a considerable study of assay parameters is necessary for the elucidation of the optimal system. Work is ongoing to exploit the results of this study for the development of an aptasensor based on a displacement-type format.

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