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Marie Brut, Ana Trapaidze, Serge Mazères, Daniel Estève, Anne Marie Gué,  
Aurélien Bancaud

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Ana Trapaidze, Marie Brut, Serge Mazères, Daniel Estève, Anne-Marie Gué , and Aurélien Bancaud



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## Thrombin detection in murine plasma using engineered fluorescence resonance energy transfer aptadimers

Ana Trapaidze,<sup>1,2</sup> Marie Brut,<sup>1,2</sup> Serge Mazères,<sup>3</sup> Daniel Estève,<sup>1,2</sup> Anne-Marie Gué,<sup>1,2</sup> and Aurélien Bancaud<sup>1,2,a)</sup>

<sup>1</sup>CNRS, LAAS, 7 avenue du colonel Roche, F-31400 Toulouse, France

<sup>2</sup>Univ de Toulouse, LAAS, F-31400 Toulouse, France

<sup>3</sup>IBPS (CNRS, IPBS (Institut de Pharmacologie et de Biologie Structurale)), Université de Toulouse, Toulouse F-31077, France

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Biodetection strategies, in which two sides of one target protein are targeted simultaneously, have been shown to increase specificity, selectivity, and affinity, and it has been suggested that they constitute excellent candidates for protein sensing in complex media. In this study we propose a method to engineer the sequence of a DNA construct dedicated to reversible thrombin detection. This construct, called Fluorescence Resonance Energy Transfer (FRET) aptadimer, is assembled with two aptamers, which target different epitopes of thrombin, interconnected with a DNA linker that contains a FRET couple and a reversible double helix stem. In the absence of target, the stem is stable maintaining a FRET couple in close proximity, and fluorescence is unquenched upon thrombin addition due to the dehybridization of the stem. We define design rules for the conception of FRET aptadimers, and develop a software to optimize their functionality. One engineered FRET aptadimer sequence is subsequently characterized experimentally by temperature scanning fluorimetry, demonstrating the relevance of our technology for thrombin sensing in bulk and diluted murine plasma. © 2015 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License.

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Aptamers constitute promising sensing elements for the detection of proteins in blood or in plasma. A large body of experimental studies has been conducted with aptamers targeted to thrombin due to the clinical relevance of this protein for therapeutic management of thrombosis. A number of specific aptamers, such as HD1, NU172, or HD22, have been used in the literature,<sup>1</sup> yet we recently showed that the poor selectivity of HD1 or NU172 was incompatible with the detection of thrombin in plasma, whereas HD22 seemed to be more selective, allowing the detection of thrombin in diluted murine plasma.<sup>2</sup> Nevertheless, the high nonspecific background encountered in complex media can be efficiently diminished by changing the detection format with double-site interaction, in particular, using sandwich assays.<sup>3–13</sup> Alternatively, the proximity between two aptamers that bind different thrombin epitopes has been exploited to collect a specific signal of the target.<sup>14–17</sup> Enhanced sensitivity and reduced reaction time have been achieved with bivalent aptamer structures in which distinct aptamers are conjugated with an optimal linker,<sup>18,19</sup> or co-conjugated to the sensor surface with an optimal density.<sup>20,21</sup> Depending on the length, design, and chemical composition of the linker, 5- to 200-fold enhancement in affinity against thrombin has been reported.<sup>18,22–26</sup>

Here we aim to take advantage of enhanced interaction specificity of bivalent aptamer structures, hereafter denoted aptadimers, to propose a sensitive, reversible, and time-

efficient strategy for thrombin optical detection in complex medium. We decided to investigate the possibility of engineering an aptadimer with HD1 and HD22 in such a way that binding to target could lead to an optical signal. We used a beacon approach, which relies on the Fluorescence Resonance Energy Transfer (FRET) effect for turning on and off the fluorescence of a fluorophore placed close to a quencher both incorporated in a DNA linker (Fig. 1(a)). The linker is engineered to form a double-helix in the absence of target, which keeps the FRET couple close to each other. Upon addition of thrombin, complementary strands of the stem dehybridize to allow interaction of the aptamers with their preferred binding sites. As a result, the distance between the fluorophore and the quencher increases and fluorescence builds up. This FRET aptadimer is reversible and compatible with mix and read mode. In the following, we describe a method to engineer the sequence of a FRET aptadimer based on a series of design rules, which are compiled in the computer-aided design software. We then validate the functionality of the selected aptadimer in the context of thrombin sensing in bulk and in plasma.

The construction of one FRET aptadimer relies on the following set of rules: (i) the length of the linker has to be sufficiently long to allow aptadimer wrapping around thrombin; (ii) the linker should have no complementarities with both aptamers and contain more thymine bases for flexibility; (iii) the stem of the linker has to be composed of complementary regions with desired melting temperature; and (iv) the quencher and fluorophore are embedded within the complementary strands of the stem opposite to each other through dye-modified thymines.

<sup>a)</sup>Author to whom correspondence should be addressed. Electronic mail: [abancaud@laas.fr](mailto:abancaud@laas.fr)

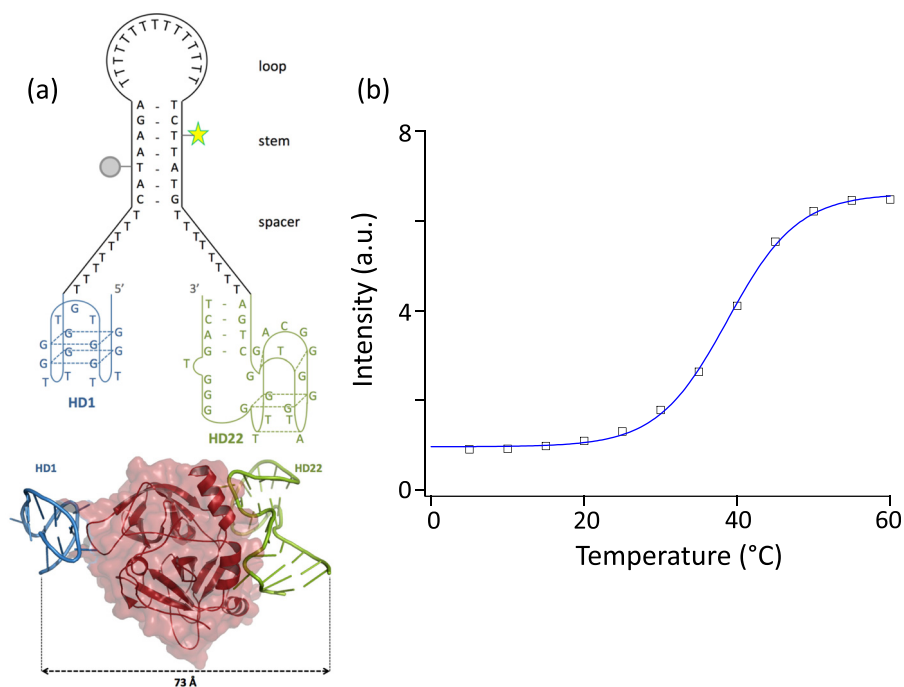


FIG. 1. Design of a FRET aptadimer. (a) The upper panel represents the sequence of the FRET aptadimer, which is composed of two aptamers (HD1 and HD22, in blue and green, respectively), two spacers, one stem of 7 base pairs, and a loop. Note the positions of the fluorophore and quencher, which are indicated by a star and a circle, respectively. In the lower panel, the crystal structure of thrombin (red) with the two aptamers HD1 (4DII pdb) and HD22 (4I7Y pdb) shown in the same colors as in the upper panel is represented with PyMol software. The distance between the lagging ends of the aptamers is 7.3 nm. (b) The dataset shows the fluorescence signal collected from the aptadimer as a function of temperature, and the corresponding fit is obtained with a sigmoidal model centered at 39°C. Note that the system was stabilized during 5 min before each fluorescence intensity measurement.

In order to clarify the first requirement, we started from the crystal structures of thrombin and its aptamers<sup>27,28</sup> in their expected orientations (lower panel in Fig. 1(a)). We measured the distance between the dangling ends of both aptamers of  $\sim 7.3$  nm (arrow in Fig. 1(a)), and considered the curvature of thrombin associated to a diameter of  $\sim 4.2$  nm in order to estimate the minimal linker length of  $\sim 10$  nm, i.e., at least 15 nucleotides (nt<sup>29</sup>). This estimation was consistent with the finding that the affinity of HD1-HD22 aptadimer interconnected with 15-nucleotide poly-adenine linker was 3-fold enhanced over that of HD1 or HD22 alone.<sup>23</sup> In addition, Ahmad *et al.* selected an optimal linker containing 35 nt by directed evolution,<sup>22</sup> and proved a  $\sim 200$ -fold enhancement in affinity.

Next, the stem of the linker represents the effector of the aptadimer. We decided to seek for complementary sequences characterized by a melting temperature of  $\sim 40$ °C. According to nearest-neighbor parameters of DNA helix formation,<sup>30</sup> this melting temperature is obtained with a stem of 5 bp, which is characterized by a free energy difference of  $\sim -4$  kcal/mol. Interaction of both aptamers with thrombin is associated to a comparatively much larger energy difference  $\sim -140$  kcal/mol,<sup>31</sup> allowing us to obtain a trade-off between stability without thrombin and facile destabilization of the stem upon interaction with the target. Finally, we wished to physically separate the different functional elements of the aptadimers using a long loop of 14 thymines and two spacers of 8 thymines between the stem and the aptamers.

We developed a software (available upon request) to define the sequence of the FRET aptadimer that matched the aforementioned criteria. We obtained a fragment of 89 nt in total with a linker of 45 nt (Figure 1(a)). We used the fluorophore ATTO-532 due to its weak variation in emission with temperature<sup>32</sup> and the corresponding quencher Black Hole Quencher 1 (BHQ1) placed at positions 47 and 26, respectively (Eurogentec). We confirmed

the folding of the linker into a double-helix stem by fluorimetry using an excitation wavelength of 510 nm and a collection window from 530–650 nm (FLSP920 spectrofluorimeter from Edinburgh Instruments, UK). A 100 nM solution of aptadimer in PBS (10 mM PB pH = 7.4, 145 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>) was heated from room temperature to 60°C by consecutive steps of 5°C separated by 5 min (dataset in Figure 1(b)). Fluorescence emission started to build up at  $\sim 25$ °C and reached its apex at 50°C, following a sigmoidal profile centered at 39°C (line plot in Fig. 1(b)). The onset between the minimum and maximum signal was more than 650%, showing the efficiency of the quenching mechanism. Furthermore, the signal decreased to its initial level as we cooled the sample to room temperature, proving the reversible pairing of the stem. Note that irreversible melting could also be triggered using the detergent Triton X-100 in the range 0.2% to 0.8% (not shown).

We subsequently checked that thrombin could be detected with our construct. We prepared solutions of aptadimers at 10, 50, and 75 nM, and titrated different concentrations of thrombin (Sigma-Aldrich) diluted in PBS (pH = 7.5) using a thrombin:aptadimer stoichiometry spanning 1:2 to 3:1. In every condition, fluorimetry was carried out in thermal scanning mode from 10 to 50°C with an increment of 5°C and 5 min of stabilization before intensity measurements (Fig. 2(a)). Fluorescence emission in the presence of increasing concentrations of thrombin expectedly augmented, because thrombin gradually destabilized the stem and in turn unquenched the fluorophore. We reasoned that thrombin could be titrated at 30°C, and plotted the relative build up in intensity *vs.* thrombin:aptadimer ratio in order to compare the different experiments on one single plot (Fig. 2(b)). Our data turned out to fall on a linear master curve that demonstrated the possibility to titrate thrombin with the designed FRET aptadimer. The slope of this curve indicated that a thrombin to aptamer molar ratio

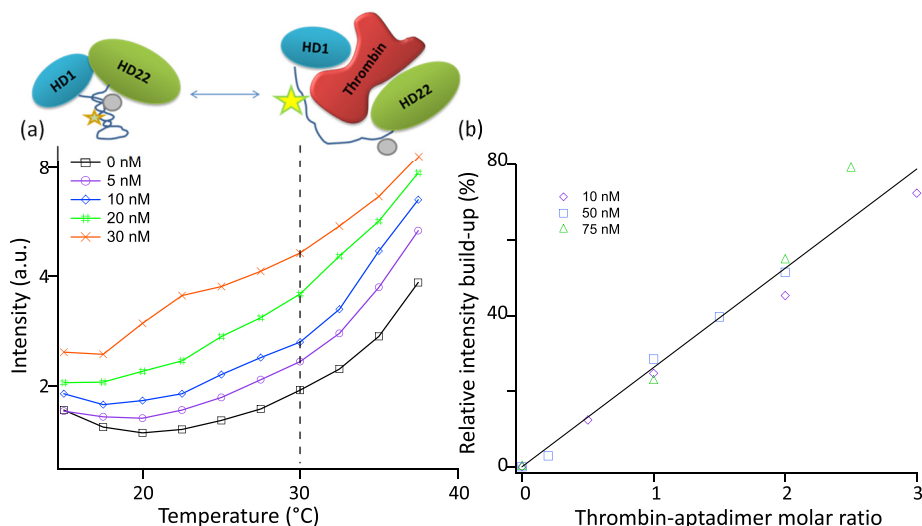


FIG. 2. Titration of thrombin in phosphate buffer. (a) The fluorescence signal is plotted as a function of temperature for a range of thrombin concentration shown in inset and a given concentration of aptadimer of 10 nM. Note that thrombin was purchased from Sigma-Aldrich (analytical grade). (b) Normalized fluorescence intensity measurements at 30 °C are reported as a function of thrombin:aptadimer molar ratio for three different aptadimer concentrations, which are shown in the inset. The line corresponds to a linear regression of the data.

of 2:1 led to an increase in fluorescence of 52%. Note that we obtained a detectable signal with 5 nM of thrombin. This lower concentration is somewhat larger than that reported with dual-reporter assays using optimal FRET couples of  $\sim 0.1$  nM,<sup>15</sup> suggesting that enhanced performances could be achieved with other fluorophores.

Finally we wished to evaluate the potential of FRET aptadimers at a concentration of 50 nM for thrombin titration in serum. Given the low selectivity of HD1 in comparison with HD22,<sup>2</sup> we wished to clarify whether the global sensing properties of the construct were sufficient to discriminate thrombin from its precursor prothrombin, which is abundantly present in serum. Temperature scanning fluorimetry of the aptadimer alone or in the presence of an equimolar concentration of prothrombin (Stago, France) yielded similar responses with marginal changes in intensity at 30 °C (Fig. 3). We then used an excess of serum albumin, which interacts with HD1 but not HD22, and did not observe any significant fluorescence augmentation. With these calibrations completed, we subsequently focused on thrombin detection in murine plasma diluted 10- and 100-fold in PBS. We observed the same response in PBS or with 1% plasma (yellow curves in Figure 3). Hence we spiked the 1% plasma

solution with 50 nM thrombin, and observed an onset in fluorescence intensity of 110%, which was larger than the signal collected with thrombin in PBS and rather corresponded to an effective thrombin concentration of 100 nM (blue datasets in Fig. 3). Given that this signal is specific to thrombin, we suggest that this response is mediated by the presence of thrombin as well as intermediate species between prothrombin and thrombin in plasma.<sup>2</sup> In any case, this high fluorescence intensity signal may also account for the large signal enhancement of more than 120% with 10-fold diluted plasma (dashed curve in Fig. 3). Residual thrombin molecules and intermediates present in 10% plasma as well as positively charged plasma components may turn on fluorescence emission, and hence compromise the sensing properties of the aptadimer. For this reason, we avoided further experiments in undiluted plasma. We note that this result was consistent with our observation that thrombin could be detected by Surface Plasmon Resonance (SPR) with HD22 in 100-fold, but not in 10-fold diluted plasma.<sup>2</sup>

Altogether we presented a method to design FRET aptadimers for recognition and quantification of thrombin. Our approach is integrated, involving (i) the development of software to define the linker sequence according to rules focused

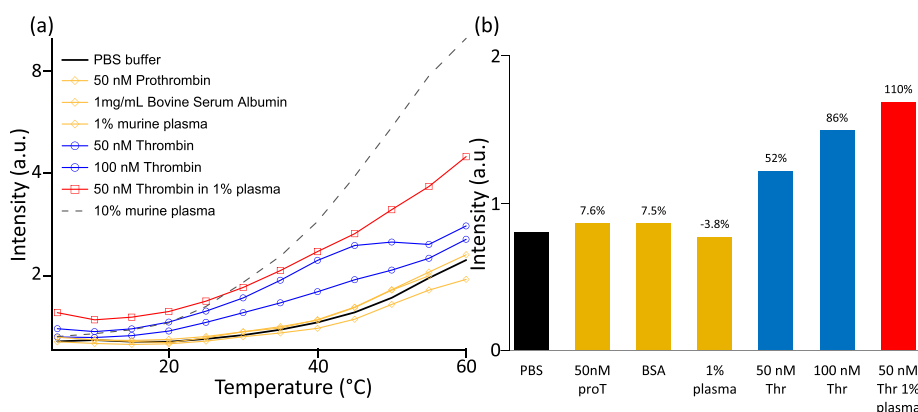


FIG. 3. Titration of thrombin in murine plasma. (a) The fluorescence signal is plotted as a function of temperature using phosphate saline buffer (pH = 7.5) or plasma, as indicated in the inset, for a concentration of aptadimer of 50 nM. The chemicals were purchased from Sigma-Aldrich, and murine plasma was provided by Sanofi (Toulouse). (b) The results are recapitulated by measuring fluorescence emission at 30 °C in every condition. Note that the experiments are color coded as in (a) with yellow, blue, and red corresponding to controls, thrombin titration in phosphate buffer, and measurements in 1% diluted plasma, respectively.

on the preservation of aptamer structure and stability, and (ii) the confirmation of aptadimer folding and functionality with FRET-based scanning temperature fluorimetry. The main advantage of this technology is associated to its ability for reversible real-time thrombin sensing, although we do not reach state-of-the-art limits of detection, which typically reach 0.1 nM or less.<sup>15</sup> Nevertheless, it would be interesting to benchmark our detection strategy with standards of the literature, such as surface plasmon resonance spectroscopy, quartz crystal microbalance, or impedance spectroscopy. For this, it may be relevant to graft aptadimers on surfaces using a thiol or biotin modified base in the loop, and to monitor the detection signal in real time with two sensing technologies. In this context, it would also be interesting to elucidate how the selectivity of aptadimers is improved over that of conventional aptamers through the use of, e.g., point mutants. In the long term, we suggest that our approach is generic, because the linker can be custom-engineered for any aptamer couple targeted to any given protein, for instance in the context of cancer with the aptamers of the human vascular endothelial growth factor<sup>18</sup> or the hepatocellular carcinoma biomarker lipocalin-2.<sup>33</sup> We thus claim that our study constitutes a step forward in the development of specific and performant aptasensors, in which the definition of molecular transducers is based on rational rules that rely on the principles of DNA nanotechnology.

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