Enzyme Assays

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Profiling of Active Thrombin in Human Blood by Supramolecular Complexes**

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The blood clotting process is characterized by the sequential activation of a series of serine proteases and cofactors, culminating in the generation of thrombin. Thrombin is a multidomain protease that induces a variety of enzymatic and cellular reactions, including conversion of fibrinogen into a fibrin clot, activation of the cofactor proteins V and VIII, and activation of platelets and endothelial cells.[1] The activity of thrombin is tightly regulated by several endogenous inhibitory mechanisms, such as the antithrombin–heparin pathway and the protein C pathway.

Clinically, impaired or unregulated thrombin formation predisposes patients to enhanced bleeding or to the development of thromboembolic complications. For example, in patients undergoing emergency or elective surgery, insufficient hemostasis caused by impaired thrombin formation may induce massive bleeding, thus threatening patients' safety and requiring treatment with blood products and expensive biologicals, such as recombinant factor VIIa.[2] On the other hand, overwhelming thrombin formation in the postoperative period is a major pathological factor contributing to the development of thrombosis. In turn, recent studies demonstrate lung embolisms to be the leading cause of death in patients undergoing elective hip and knee replacement surgery, despite the widespread use of prophylactic anticoagulant treatment.[3] These examples document that plasma levels of free thrombin represent a promising biomarker reflecting a patient's individual hemostatic status to guide successful treatment decisions. However, no diagnostic assay is available to date that enables the direct measurement of thrombin concentrations in circulating blood from patients. Indeed, thrombin generation is indirectly determined as antithrombin–thrombin complexes (TAT). However, owing to the long half-life of TAT compared to thrombin, the concentration of these complexes in blood samples does not reflect the coagulation status of patients accurately.

Aptamer-based biosensor systems, so-called aptasensors, represent a promising format that allows the detection of biomarkers.[4] Owing to the ease of use of two well-known DNA aptamers that recognize thrombin, namely HD1 and HD22, a variety of such sensors have been described to measure thrombin, but none of them has been validated and proven useful in daily clinical practice.[5] One reason might be the limited knowledge of preanalytical conditions necessary to avoid rapid thrombin inactivation by endogenous inhibitors as observed ex vivo. We therefore developed a supramolecular approach that overcomes these limitations. The assay allows measurement of the in vivo coagulation status reflected by thrombin concentrations found in the drawn blood from patients. By studying normal individuals and patients undergoing hip-replacement surgery, we demonstrate that this assay allows close-mesh monitoring of the activity level of the coagulation system under clinical conditions.

We use the recently described bivalent aptamer HD1-22, which simultaneously targets both exosites of thrombin.[6] The aptamer binds with sub-nanomolar affinity and with high selectivity to thrombin, as exemplified by a 100-fold decreased affinity to prothrombin. These characteristics allow us to hypothesize whether HD1-22 could be useful in the development of a clinically applicable assay format for quantification of thrombin in patient plasma samples. Owing to the selectivity of the aptamer, this task might be possible even in the presence of a high molar excess of prothrombin, as found in native blood. After capturing, thrombin will be visualized by its amidolytic activity, as the interaction of HD1-22 with thrombin leaves the active site functional and accessible for small peptide substrates. This enzyme-capture format combines the sophisticated binding properties of the aptamer with the high sensitivity and specificity of enzyme-catalyzed reactions. The general principle of this oligonucleotide-based enzyme capture assay (OECA) is shown in Scheme 1 (see the Supporting Information for details on assay performance and validation).

Blood to be analyzed is drawn into tubes containing citrate and the reversible active-site thrombin inhibitor argatroban to prevent ex vivo complex formation between thrombin and its physiological inhibitors (Figure 1 a). The use of argatroban was shown to be superior to the use of the broad

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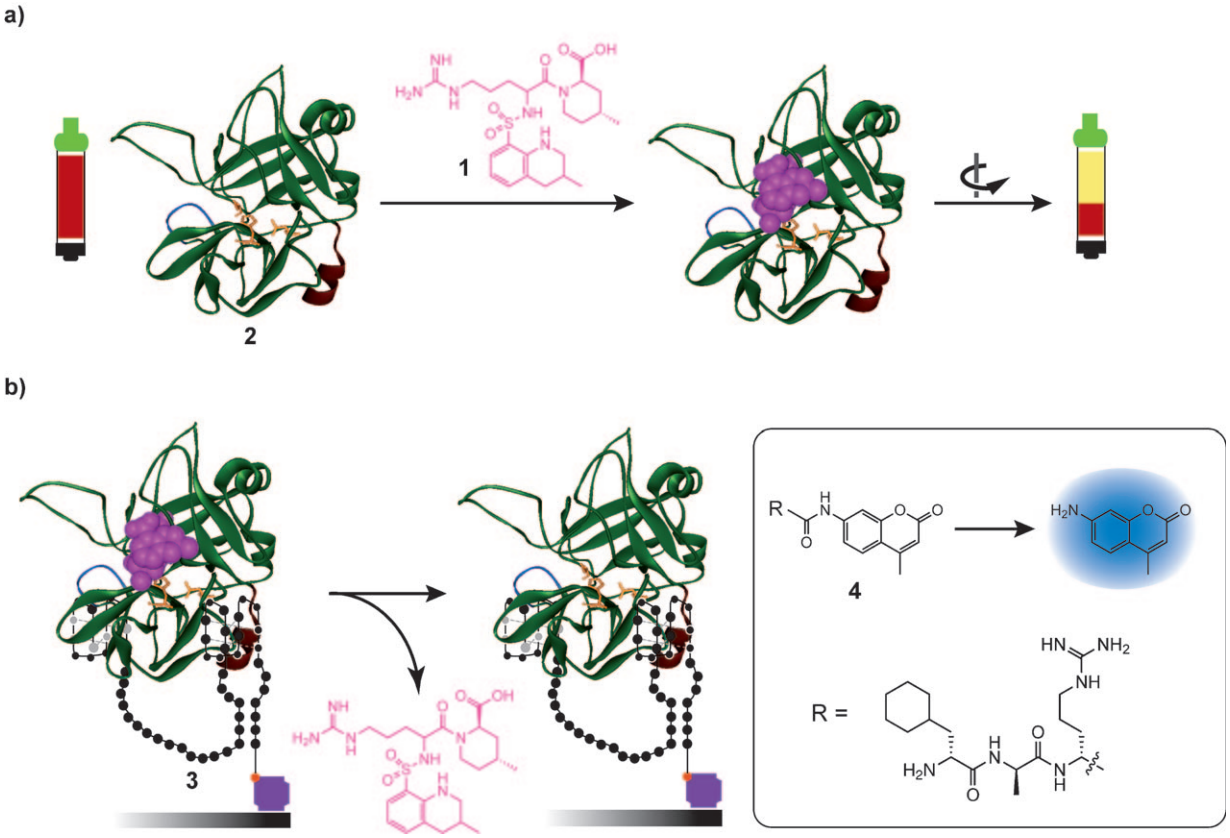

Scheme 1. General principle of the oligonucleotide-based enzyme capture assay for thrombin measurement. a) During the blood sampling process, an anticoagulant buffer (citrate) containing the reversible active-site inhibitor argatroban (**1**) is added to the blood sample containing thrombin (**2**, PDB 1DWC). Complex formation between argatroban and thrombin efficiently prevents irreversible inhibition of thrombin by endogenous thrombin inhibitors. b) Wells of streptavidin-coated microtiter modules previously loaded with the 3'-biotinylated anti-thrombin aptamer HD1-22 (**3**) that simultaneously targets exosites I and II of thrombin are overlaid with plasma. After incubation and capturing of the argatroban-thrombin complex by HD1-22, wells are washed to remove plasma remains and reversibly bound argatroban. Subsequently, a thrombin-specific peptide substrate bearing an AMC (7-amino-4-methylcoumarin) fluorogenic probe (H-D-CHA-Ala-Arg-AMC, **4**) is added to quantitatively determine the amount of functional active thrombin captured in the wells.

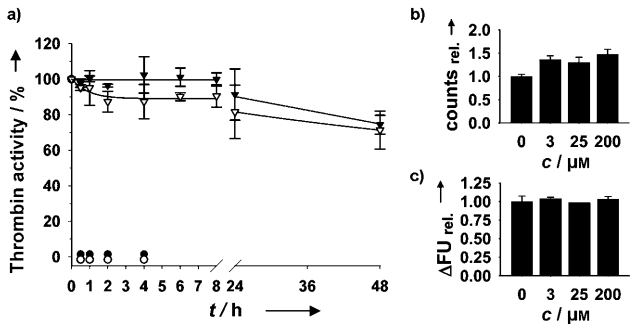

Figure 1. a) Evaluation of preanalytical conditions. Thrombin was added to citrate anticoagulated whole blood in the presence (triangles) or absence (circles) of argatroban (100 μM). Blood samples were stored at room temperature (closed symbols) or on ice (open symbols) until analyzed. b,c) Influence of reversible active-site inhibitors on binding of aptamers to thrombin. Interaction of 5′-radiolabeled and 3′-biotinylated aptamer HD1-22 with thrombin (5 nM) in the presence of increasing concentrations of argatroban (b) and the effect of argatroban on binding of target enzymes to immobilized aptamers (OECA setting; c). Increasing concentrations of argatroban did not influence the assay outcome with respect to the detection of thrombin at 1 ng mL^{-1} concentration levels.

protease inhibitor benzamidine (Supporting Information Figure S1).[7] After appropriate storage, the tubes were centrifuged, and plasma was either directly tested or stored at −40 °C until assayed (Supporting Information Figure S2). Binding of argatroban does not hamper aptamer–thrombin recognition; indeed, interaction was found to be favored in the presence of argatroban (Figure 1 b), thus allowing sequestration of thrombin from plasma samples using 3′-biotinylated HD1-22 variants immobilized on streptavidin-coated microtiter modules. For kinetic reasons and because of its lower affinity to thrombin, argatroban can be removed efficiently by washing after sequestration, whereas the complex between HD1-22 and thrombin remains stable.[6b,8] In this way, the active site will be made amenable again for selective fluorogenic peptide substrates to visualize thrombin activity (Figure 1 c).

The OECA showed a dynamic range from low to high picomolar concentrations. In detail, the calculated lower limit of quantification (LLOQ) was determined to be (0.039 ± 0.019) ng mL^{-1} ((1.08 ± 0.53) pM), and the limit of detection (LOD) was calculated to be (0.017 ± 0.004) ng mL^{-1} ((0.47 ± 0.11) pM, Figure 2 a). Within- and between-run coefficients of

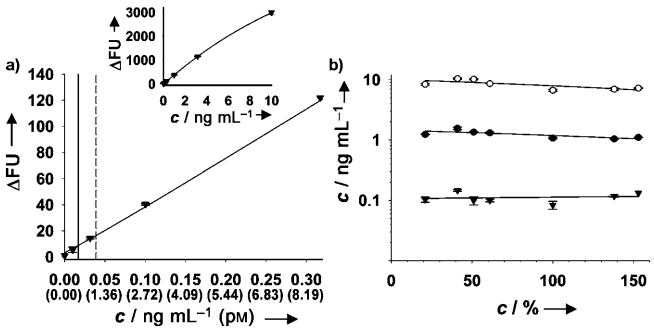

Figure 2. a) Dynamic range and sensitivity of the OECA. The lower concentration ranges of a typical standard curve obtained with plasma-based thrombin calibrators are shown. The vertical solid and dashed lines represent the LOD and LLOQ, respectively. The inset shows the full range of the applied standard curves (0–10 ng mL^{-1}). All data points were interpolated by a four-parameter logistic function. b) Influence of prothrombin concentrations on assay outcome. To test the influence of prothrombin on the sensitivity of thrombin detection, plasma samples containing the indicated concentrations of prothrombin (*x* axis) were spiked with thrombin to achieve final concentrations of 8 ng mL^{-1} (○), 1 ng mL^{-1} (●), and 0.1 ng mL^{-1} (▼), and recovery rates were measured. Results are expressed as mean ± SD (SD = standard deviation >) of three independent experiments.

variation did not exceed 10% even for the lowest concentration of thrombin tested (Table 1). To investigate prothrombin influence on assay performance, we analyzed thrombin-spiked plasma samples containing prothrombin concentrations ranging from very low to highly pathological concentrations. The results show that only the recovery rates of higher concentrations of thrombin were slightly influenced by prothrombin, whereas in all other cases no influence was observed (Figure 2b). These data emphasize the high specificity of the OECA.

Table 1: Reproducibility of thrombin detection with OECA.

Input concentration [ng mL^{-1} (pM)]	Overall found [ng mL^{-1} ± SD (pM)]	Mean within-run CV[a] [%]	Between-run CV [%]
5.0 (136)	4.73 ± 0.26 (129)	7.55 ± 5.99	5.55
1.0 (27.2)	0.89 ± 0.04 (24.3)	6.47 ± 3.45	4.01
0.1 (2.72)	0.09 ± 0.01 (2.45)	6.60 ± 4.07	10.03

[a] CV = coefficient of variation.

In vivo the activity of thrombin is strongly controlled by a variety of activating and inhibitory mechanisms, including inactivation of thrombin by complex formation with antithrombin or binding to cell-surface receptors such as thrombomodulin. Therefore, it was unclear whether free thrombin circulates in human blood under physiological conditions. When plasma samples obtained from 20 healthy blood donors were studied, free thrombin was detectable, but levels were found to be extremely low and therefore fell short of the LLOQ or even the LOD of the assay (Supporting Information Figure S3). These findings are in accordance with the general consideration that the hemostatic system is tightly regulated under physiological conditions.

To study if and to what extent activation of the clotting cascade increases the plasma level of thrombin, we analyzed blood samples from patients undergoing total hip arthroplasty. This type of surgery is characterized by a high degree of standardization resulting in comparable tissue damage assuming a similar degree of trauma-associated activation of the clotting system. Increased plasma levels of thrombin were detectable during the course of operation, reaching peak values above 100 pM (Figure 3a). As an additional, albeit indirect, measure of thrombin formation we determined thrombin–antithrombin complexes (TAT) that are generated during inactivation of thrombin by antithrombin. In general, plasma levels of thrombin correlated well with TAT levels (Figure 3b). Interestingly, grouping and correlation of individual data sets revealed a high interindividual variability of procoagulant responses (Figure 3c). Moreover, values of thrombin measured during individual surgeries correlate only poorly with that of TAT complexes (Figure 3c). This result can be explained by differences in the circulating half-lives times of thrombin and TAT. While the half-life of TAT is less than 15 min,[9] the half-life of thrombin can be expected to

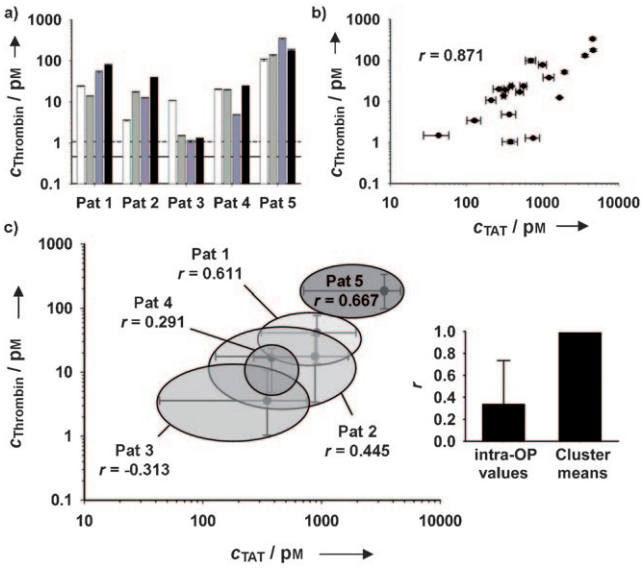

Figure 3. Intraoperative monitoring of thrombin generation. a) Blood samples of five patients undergoing elective hip replacement surgery were obtained after anesthesia induction, after joint extraction, after artificial joint implantation, and during skin closure (see the Supporting Information for details). Values for thrombin determined by OECA are shown in this order from left to right. The dashed and solid horizontal lines represent the LLOQ and LOD of the assay, respectively. b) Overall correlation of thrombin with TAT complexes. c) Means and correlations of TAT complexes and thrombin concentrations determined in the four samples obtained during each individual surgery. The error bars represent the min–max range, while the ovals demarcate individual patient clusters only for illustration purposes without stressing any statistical interpretations. Mean correlations of intraoperative values and correlations of cluster means are summarized in the inset of (c).

be less than 1 min (Supporting Information Figure S4). In this way, detection of thrombin more accurately reflects the current state of procoagulant activity, whereas TAT levels rather reflect the average stage of thrombin generation over a longer period of time.

Herein, we demonstrate that the application of argatroban and the aptamer HD1-22 allows the development of a supramolecular oligonucleotide-based enzyme capture assay for the sensitive detection of thrombin in correspondingly primed plasma and thus under routine clinical conditions. We optimized the pre-analytical settings necessary for the measurement of thrombin plasma levels and demonstrate that thrombin circulates at sub-picomolar concentrations under physiological conditions. Furthermore, we were able to monitor trauma-associated procoagulant responses of the coagulation pathway in patients undergoing major orthopedic surgery. In this way we make the key enzyme of this network—thrombin—directly measurable.

We propose that direct determination of plasma levels of the active reaction partners of complex and dynamic biological networks such as the coagulation cascade more accurately reflect disease-relevant intermediate phenotypes and therefore represent a better basis for treatment decisions, consequently improving patient care and outcomes. Because of its pivotal role within the coagulation network, thrombin levels in plasma are hypothesized to be a valuable enzyme biomarker that might fulfill these requirements. The OECA platform presented herein demonstrates a possible route for the direct measurement of active coagulation factors in human plasma samples. Furthermore, the results provide substantial data that build a profound basis for future assay developments. We thus strongly believe that routine clinical application of the described assay will enhance patient care.

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