

Monitoring Thrombin Generation by Electrochemistry: Development of an Amperometric Biosensor Screening Test for Plasma and Whole Blood

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BACKGROUND: Complete investigation of thrombophilic or hemorrhagic clinical presentations is a time-, apparatus-, and cost-intensive process. Sensitive screening tests for characterizing the overall function of the hemostatic system, or defined parts of it, would be very useful. For this purpose, we are developing an electrochemical biosensor system that allows measurement of thrombin generation in whole blood as well as in plasma.

METHODS: The measuring system consists of a single-use electrochemical sensor in the shape of a strip and a measuring unit connected to a personal computer, recording the electrical signal. Blood is added to a specific reagent mixture immobilized in dry form on the strip, including a coagulation activator (e.g., tissue factor or silica) and an electrogenic substrate specific to thrombin.

RESULTS: Increasing thrombin concentrations gave standard curves with progressively increasing maximal current and decreasing time to reach the peak. Because the measurement was unaffected by color or turbidity, any type of blood sample could be analyzed: platelet-poor plasma, platelet-rich plasma, and whole blood. The test strips with the predried reagents were stable when stored for several months before testing. Analysis of the combined results obtained with different activators allowed discrimination between defects of the extrinsic, intrinsic, and common coagulation pathways. Activated protein C (APC) predried on the strips allowed identification of APC-resistance in plasma and whole blood samples.

CONCLUSIONS: The biosensor system provides a new method for assessing thrombin generation in plasma or whole blood samples as small as 10 μ L. The assay is easy to use, thus allowing it to be performed in a point-of-care setting.

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Hemostasis is based on the complex interaction between vessel wall, blood cells, and coagulation proteins. The investigation of hemostatic disorders requires a stepwise approach. It includes patient history, physical examination, and laboratory tests such as coagulation assays, fibrinolysis tests, and platelet function assays. Carrying out a complete investigation of a thrombophilic or hemorrhagic clinical presentation is a time-, apparatus-, and cost-intensive process. Sensitive screening tests that provide a complete overview of the function of the hemostatic system, or defined parts of it, would be more useful than individual specific tests for the different components, because such tests would allow the development of a cost-effective diagnostic strategy.

Information obtained from global coagulation assays, such as prothrombin time and activated partial thromboplastin time, does not cover all aspects of the hemostatic processes and is limited to the initial phase of blood coagulation. At the time of fibrin polymerization, the endpoint of coagulation assays, just 5%–10% of prothrombin has been converted to thrombin; further progress of prothrombin conversion into thrombin is not considered (1). An old method taking into account the phase of coagulation propagation, fibrin polymerization, and fibrinolysis is thrombelastography (2). It can be used for whole blood, anticoagulated whole blood, and platelet-rich or platelet-poor citrated plasma. It monitors hemostasis under low shear conditions as an overall dynamic process instead of revealing information about isolated parts of the different pathways.

For the past several years, there has been a renewed awareness of the significance of thrombin generation (TG)³ for the evaluation of coagulation disorders (1, 3). Several publications have shown that monitoring TG is a valuable research tool and has the potential to become a useful method for diagnosing hypo- and

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³ Nonstandard abbreviations: TG, thrombin generation; APC, activated protein C; F, factor; HMWK, high-molecular-weight kininogen; PK, prekallikrein; CAT, Calibrated Automated Thrombogram; TGA, Technothrombin Kit; ETPa, Endogenous Thrombin Potential Assay; inTDT, Thrombin Dynamics Test.

hypercoagulability and monitoring patient care. Thrombin generation assays measure time-dependent changes in thrombin concentrations in platelet-rich or platelet-poor plasma samples, even after clot formation. More than 50 years ago, a subsampling technique for measuring TG was described (4). In 1993, Hemker et al. (5) introduced a procedure for continuous monitoring of thrombin concentration by means of a chromogenic substrate, followed a few years later by an automated method for monitoring TG by continuous fluorescent measurement (6–8). The feasibility of continuous measurement of TG by chromogenic or fluorogenic methods has considerably simplified the process. These methods have limitations for practical application, however, owing to laborious preparation of the reagent mixture, relatively voluminous equipment, and requirement of blood plasma.

In the field of blood glucose monitoring, electrochemistry is an established method. The electrochemical principle of enzyme activity detection has now been made possible for thrombin, as well. We present a new biosensor screening test that monitors the formation of thrombin by electrochemical detection on single-use test strips.

Materials and Methods

AMPEROMETRIC BIOSENSOR SYSTEM FOR THROMBIN

We developed a biosensor system for thrombin that consists of single-use electrochemical sensors in the shape of a strip and a potentiostatic measuring unit connected to a personal computer. The sensor strips (test strips) are electrically connected to the measuring unit by means of a support device that additionally works as a thermostat. The measuring unit applies a constant potential to the working electrode in the sensor strip, leading to a redox reaction at electroactive compounds (9). According to the law of Faraday, the electric current monitored is a quantitative measure of the analyte (10). More precisely, blood is added to a specific reagent mixture immobilized in dry form on the strip, including an activator substance of the blood coagulation system and an amperogenic substrate specific for thrombin. The generated thrombin cleaves the substrate, producing a leaving group that creates an amperogenic signal, i.e., an electric current whose intensity is monitored as a function of time (chronoamperometry). At a potential of 300 mV, the leaving group meets the necessary conditions, such as good solubility in an aqueous medium, high signal response at low potential, and linear dependence of the electric current intensity on its concentration (11). For the prototype setting, the potential is controlled by the potentiostatic apparatus Potentiostat PGP 201 (Radiometer), which, for recording the electric signal, is con-

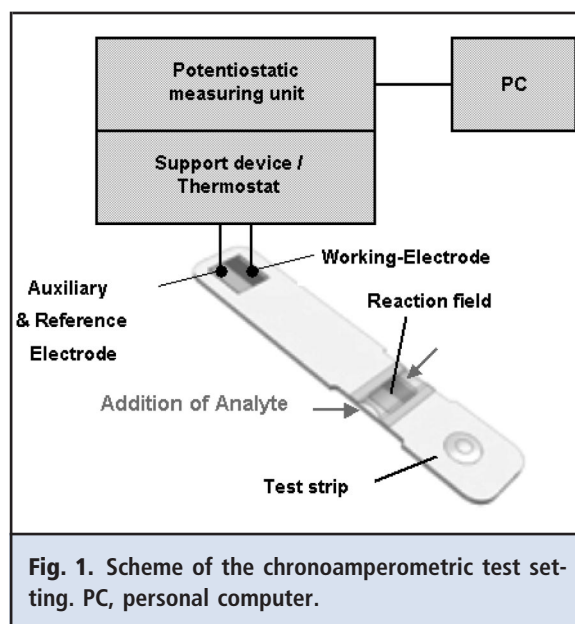


Fig. 1. Scheme of the chronoamperometric test setting. PC, personal computer.

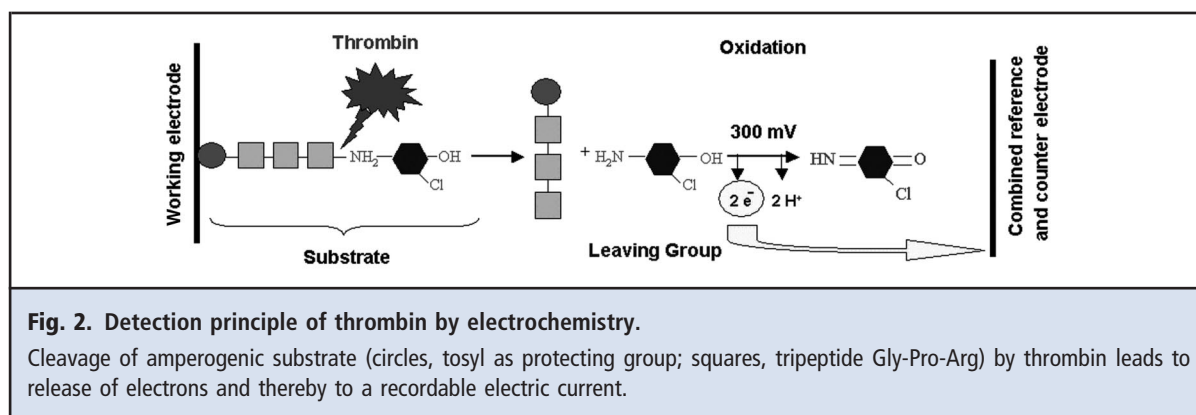
nected to a personal computer supplied with the software Volta Master 1 (Radiometer Analytical SA). The sensor strips and the support device are designed and fabricated by Asulab. The measurement principle, amperogenic substrate, and design of the test strips are the subject matter of several patents (12–14).

SETTING OF THE BIOSENSOR SYSTEM

All amperometric measurements are performed on a sensor strip (40 by 8 by 0.46 mm) (microelectrode) consisting of an isolating thin support, a palladium electrode as working electrode, and an Ag/AgCl electrode as a combined reference and counterelectrode. There is a first open field (Fig. 1, “reaction field”) for the placement of the reagent mixture and a second open field for the connection to the potentiostatic measuring unit. The surface of the working electrode in the reaction field is defined as 0.054 cm² and is covered with an activator of coagulation predried on that surface (see below). To prevent the sample from evaporating, the reaction field is protected by a concave plastic cover. Two small lateral apertures allow sample application from either side by means of capillary force. A scheme of the test setting is shown in Fig. 1.

REAGENT MIXTURES

The main ingredients of the reagent mixture immobilized on the reaction field of the test strip are an activator substance, further additives assisting the coagulation process, and an amperogenic substrate for thrombin. For activation of the plasmatic coagulation cascade, the test strips contain either an extrinsic or an



intrinsic activator substance. Test strip A contains a commercial thromboplastin reagent as source of tissue factor. Test strips B and C contain a nonphysiological contact phase activator, e.g., silica. Additionally, test strip C contains activated protein C (APC) (Coatest APC Resistance; Chromogenix/Instrumentation Laboratory). To assist the coagulation process *in vitro*, phospholipids (1,2-diacyl-sn-glycero-3-phospho-L-serine from bovine brain; Sigma-Aldrich) and Ca^{2+} are added to each type of sensor strip. Tos-Gly-Pro-Arg-4-Amino-2-chlorophenol AcOH (Pentapharm) is used as amperogenic substrate (US patent 6495336). It is selectively cleaved by thrombin (13). The central part is a tripeptide sequence (Gly-Pro-Arg), a protecting group (tosyl) is coupled to the N-terminus, and an amperogenic leaving group (4-amino-2-chlorophenol AcOH) is coupled to the C-terminus of the tripeptide.

MEASUREMENT PROCEDURE

Sensor strips are inserted into the measuring unit connected to the thermostat, which keeps measurement temperature at 37 °C. Chronoamperometry is performed after addition of 8 μL of a 1:4 dilution of blood sample in working buffer (Hepes 20 mmol/L, NaCl 140 mmol/L, 5 g/L bovine serum albumin; pH 7.35) to the reaction field of test strips A and B. Whole blood samples are prediluted 9:10 with trisodium citrate 106 mmol/L. (The samples are not diluted for measurements with test strip C.) During reaction of the measurement process, the thrombin formation is displayed as actual electric current (nanoamperes, or nA). The software program automatically computes the current as $\mu\text{A}/\text{cm}^2$ (taking into account the surface of the working electrode on the reaction field of the test strip) and the personal computer displays the corresponding curve. The measurement process is stopped when the current curve reaches its peak value, or at the latest after 30 min.

SAMPLES

Because electrochemical detection of thrombin formation is not affected by color or turbidity, a broad range of sample types is applicable: platelet-poor plasma, platelet-rich plasma, and whole blood samples (13). To validate the method, we performed measurements with thrombin (Dade Behring) and several commercially available coagulation factor-deficient plasmas [factor V (FV)-, FVII-, FVIII-, FIX-, FX-, FXI-, and FXII-deficient plasma (Dade Behring); high-molecular-weight kininogen (HMWK)-deficient and prekallikrein (PK)-deficient plasma (George King Bio-Medical)]. As normal citrated plasma, we used a pool plasma of several hundred healthy donors (obtained from CSL Behring). We also compared the effect of plasma samples of subjects with APC resistance caused by heterozygous FV R506Q mutation with the normal pool plasma sample, to investigate the influence of APC on test strip C. To demonstrate the applicability of the method to whole blood, we performed measurements with different capillary whole blood samples, i.e., whole blood from healthy donors and APC-resistant whole blood.

Results

CURVE PROGRESSION

The generated thrombin cleaves the amperogenic substrate, which oxidizes at 300 mV and releases electrons leading to an amperometric signal (Fig. 2). The raw data are exported to Microsoft Excel. The intensity of the electric current reflecting thrombin formation is expressed as $\mu\text{A}/\text{cm}^2$ over time. Several parameters characterize the observed curve (Fig. 3A): lag time [t-lag(s)], time to peak [t-peak (s)], maximal current [I-max($\mu\text{A}/\text{cm}^2$)], maximal slope of the electric current course [S-max (current per time)], and time to S-max [tS-max (s)]. The intensity of the electric current is related to the amount of active thrombin present in the reaction field. Fig. 3B shows curves generated by increas-

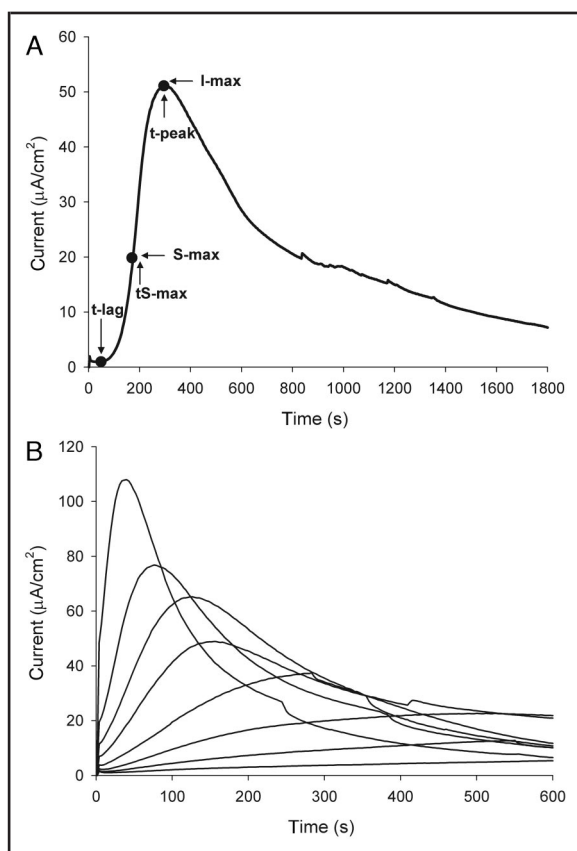


Fig. 3. Progression of thrombin's chronoamperometric curve.

(A), Thrombin generation curve in normal platelet-poor plasma with indication of curve-specific parameters; lag time (t-lag), maximal slope (S-max), time to maximal slope (tS-max), maximal current (I-max), and time to peak (t-peak). (B), Chronoamperometric curves generated by different thrombin concentrations, i.e., thrombin 50 U/mL (highest peak); diluted 1:2, 1:4, 1:8, etc., down to 1:128 (flattest curve) in working buffer; measurements performed on test strip A (lot A2).

ing amounts of purified thrombin, ranging from 0.4 to 50 U/mL. For instance, as shown in Supplemental Figs. 1–3 (which accompany the online version of this article at <http://www.clinchem.org/content/vol55/issue3>), the amount of thrombin present in the sample directly correlates with the maximal positive slope of the curve, S-max (by linear correlation, $R^2 = 0.995$), with the time to reach the peak of maximal current, t-peak (by power function, $R^2 = 0.9573$), and the maximal current, I-max (by logarithmic correlation, $R^2 = 0.9634$).

MEASUREMENTS IN PLATELET POOR PLASMA

To evaluate the reproducibility of the test system, we repeated several measurements with the same normal

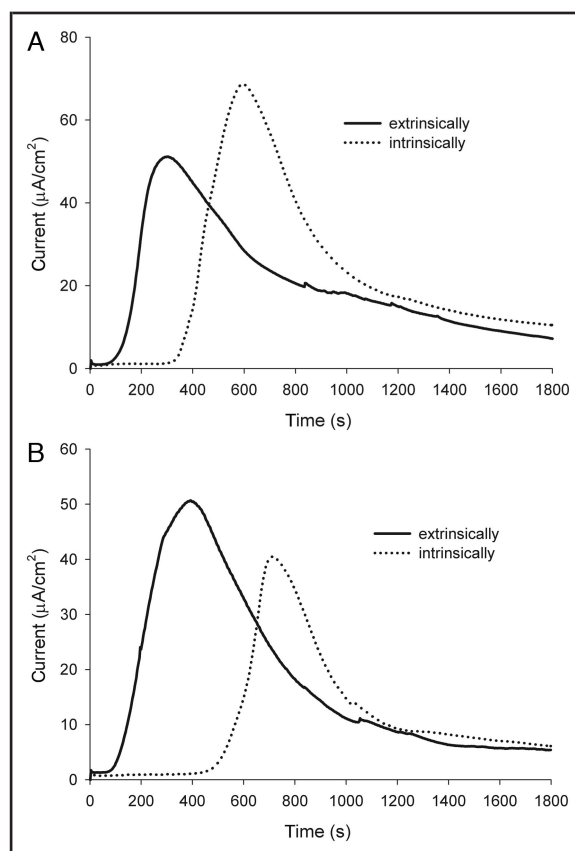


Fig. 4. Electrochemistry allows measurements of thrombin generation in plasma and whole blood.

(A), Normal plasma activated by tissue factor [straight line; test strip A (lot A1)] and contact phase activator [dotted line; test strip B (lot B1)]. (B), Capillary whole blood activated by tissue factor [straight line; test strip A (lot A2)] and contact phase activator [dotted line; test strip B (lot B2)].

pooled citrated plasma sample. Fig. 4A shows typical curves when normal plasma is activated by tissue factor (test strip A, straight line) or silica (test strip B, dotted line). The lag time until thrombin generation detected [mean (SD)] lasts 62 (6) s (CV 9.7%) with test strip A ($n = 5$) and 331 (15) s (CV 4.5%) with test strip B ($n = 6$). The peak of thrombin formation [51.6 (2.7) $\mu\text{A}/\text{cm}^2$ (CV 5.2%) vs 66.8 (14.0) $\mu\text{A}/\text{cm}^2$ (CV 21.0%)] is attained after 303 (8) s (CV 2.6%) and 641 (58) (CV 9.0%) s with test strips A and B, respectively.

MEASUREMENTS IN WHOLE BLOOD

We also evaluated the performance of the method with whole blood. For this purpose, we made multiple measurements with the same citrated capillary whole blood sample. Samples of normal capillary whole blood mea-

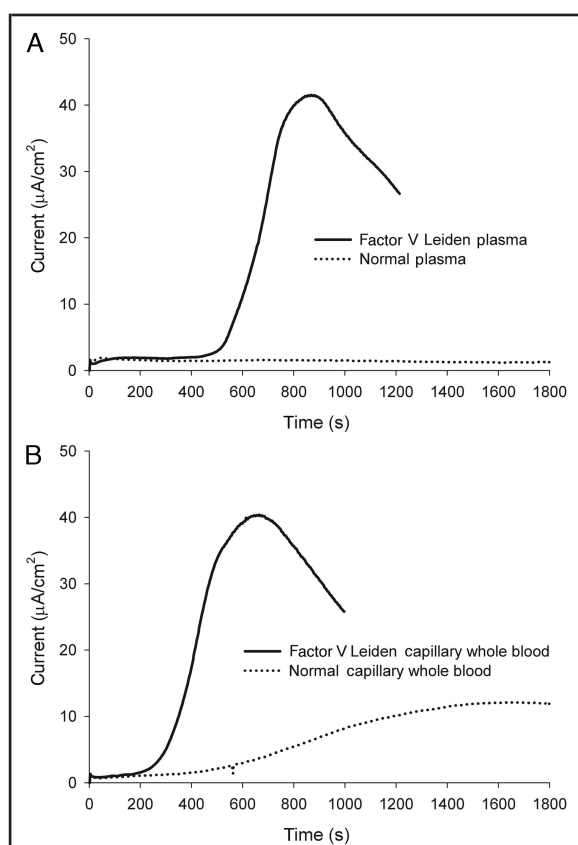


Fig. 5. Detection of APC resistance by addition of APC to the reaction mixture on the test strips.

(A), Discrimination between platelet-poor plasma samples of a patient with a heterozygous factor V Leiden mutation and platelet-poor pooled normal plasma sample. (B), Discrimination between whole blood samples of a patient with a heterozygous factor V Leiden mutation and a normal whole blood sample. Measurements performed on test strip C (lot C1).

sured with test strips A and B give signals similar to those of normal plasma samples (Fig. 4B). After activation by a tissue factor (test strip A), t-lag is 64 (3) s (CV 4.7%). The peak of 52.0 (1.2) $\mu\text{A}/\text{cm}^2$ (CV 2.3%) is reached at 378 (14) s (CV 3.7%) ($n = 3$). Measured on test strip B, t-lag is 460 (40) s (CV 8.7%), t-peak 758 (59) s (CV 7.8%), and I-max 35.4 (6.9) $\mu\text{A}/\text{cm}^2$ (CV 19.5%) ($n = 5$). Neither the turbid red color nor the cells in whole blood substantially interfered with the chronoamperometric signal.

DETECTION OF RESISTANCE TO APC

As shown in Fig. 5A, normal pooled plasma measured on test strips containing a defined mixture of APC and

a contact activator (test strip C) did not lead to any measurable electric current. In contrast, platelet-poor plasma samples of an APC-resistant patient produced a clear signal. As for capillary whole blood tested on test strips C, a normal sample led to a slowly rising curve, whereas capillary whole blood from a patient with a factor V Leiden mutation led to a relatively fast rising and high signal (Fig. 5B).

PERFORMANCE OF THE METHOD

Comparing the results of patient samples with the reference values derived from healthy individuals gives information about possible coagulation factor deficiencies. As proof of concept, Table 1 summarizes results obtained with several clotting factor-deficient plasmas. Combining 2 different types of sensor strips makes it possible to discriminate between disturbances located in extrinsic, intrinsic, and common pathways of the coagulation cascade. In FII-, FV-, and FX-deficient plasma, thrombin formation is suppressed with both activators. Testing FVII-deficient plasma, extrinsic thrombin formation is suppressed, whereas the intrinsic curve is normal. In plasma samples deficient in coagulation factors of the intrinsic system (FVIII, FIX, FXI, FXII, HMWK, PK), only thrombin formation after contact phase activation is reduced or (in the case of FXII-, HMWK-, and PK-deficient plasma) even suppressed.

Discussion

This biosensor system enables monitoring of thrombin generation in an easy, electrochemical manner. Since Hemker et al. (5) developed a procedure allowing for the first-time continuous monitoring of TG, research efforts have remained limited to optical detection. The test system developed by Hemker is commercialized as "Calibrated Automated Thrombogram" (CAT) by Thrombinoscope BV. Since the introduction of the CAT, more or less similar systems (or reagent kits) for measuring TG have been marketed by other companies, such as Technothrombin Kit TGA by Technoclone GmbH (15), Endogenous Thrombin Potential Assay (ETPa) by Siemens Healthcare Diagnostics Inc., and Pefakit Thrombin Dynamics Test (inTDT) by Pentapharm (16). All these systems are based on optical detection methods. The 4 techniques are divided into 2 chromogenic methods (ETPa and inTDT) and 2 fluorogenic methods (CAT and TGA). Furthermore, they differ in how the coagulation cascade is activated and in the final interpretation of the monitored curve.

The electrochemical method presented here is based on the same principle as conventional procedures. The plasma coagulation is activated in vitro ei-

Table 1. Plasma deficient of coagulation factors compared with plasma from healthy individuals and normal pool plasma.^a

Plasma	Extrinsic activation (test strip A, lot A1)				Intrinsic activation (test strip B, lot B1)			
	n	t-lag, s	t-peak, s	I-max, $\mu\text{A}/\text{cm}^2$	n	t-lag, s	t-peak, s	I-max, $\mu\text{A}/\text{cm}^2$
From healthy individuals	5	47 (5)	269 (27)	45.9 (1.4)	5	271 (36)	518 (34)	71.8 (8.5)
Normal pool plasma (frozen)	5	62 (6)	303 (8)	51.6 (2.7)	6	331 (15)	641 (58)	66.8 (14.0)
FII-deficient	3	>1800	>1800	NA ^b	3	>1800	>1800	NA
FV-deficient	3	>1800	>1800	NA	3	>1800	>1800	NA
FVII-deficient	3	>1800	>1800	NA	4	320 (18)	642 (47)	69.9 (9.0)
FVIII-deficient	3	59 (5)	308 (58)	37.2 (6.0)	3	520 (13)	1259 (167)	22.6 (7.8)
FIX-deficient	3	67 (9)	307 (23)	37.1 (4.3)	3	606 (87)	1365 (241)	16.6 (1.3)
FX-deficient	3	>1800	>1800	NA	3	>1800	>1800	NA
FXI-deficient	3	62 (10)	290 (10)	38.0 (8.0)	3	595 (13)	1033 (84)	22.8 (3.6)
FXII-deficient	3	59 (4)	308 (30)	44.3 (2.0)	3	>1800	>1800	NA
HMWK-deficient	3	63 (5)	235 (5)	41.5 (2.1)	3	>1800	>1800	NA
PK-deficient	3	56 (8)	260 (6)	42.7 (0.8)	3	>1800	>1800	NA

^a Data are mean (SD).

^b NA, not applicable [because no peak was detected during measuring time (30 min)].

ther extrinsically or intrinsically, whereupon the generated thrombin selectively cleaves an oligopeptide. The amount of the amperogenic leaving group depends on the amount of thrombin generated. Instead of a fluorescence change or the release of a chromophore, however, thrombin generated in our system leads to an electric current flow, which is recorded amperometrically. Besides this difference in the measuring method, the most obvious difference of this method vs the traditional methods is the test setting. The electrochemical principle, which is well established in the field of blood glucose monitoring (10), enables miniaturization of the test setting to a biosensor system consisting of a measuring device and single-use test strips. The test strips containing the predried reagents can be produced in ready-to-use batches, thus minimizing time-consuming handling before running a test. Individually packed under a dry, inert atmosphere, the test strips may be stored for several months or, depending on the reagents, even for years (unpublished observations). For an individual measurement, only 2 μL (for test strips A and B) or 8 μL (for test strip C) of a sample are required, a considerably smaller volume than is necessary for conventional optical systems (40 μL for TGA Technoclone, 60 μL for inTDT Pentapharm, 80 μL for CAT Thrombinoscope, 110 μL for ETPa Dade Behring). Probably the most interesting advantage of the present biosensor system is the possibility of determining TG in platelet-poor plasma, platelet-

rich plasma, and whole blood. The amperogenic leaving group reflects in a physicochemical manner the formation of thrombin. Thus, the electric signal is disturbed by neither the appearance of a clot nor the presence of platelets, nor is it affected by the color of the sample. Running a calibrator with each sample, to compensate for color of the blood sample and for the so-called inner-filter effect (nonlinearity of fluorescence intensity with increasing concentration of fluorescent molecules) (17) is not required. The possibility of making measurements in whole blood samples opens new avenues in the research and in the clinical field as well. In fact, using whole blood may allow point-of-care testing, and, at the same time, with whole blood the testing conditions come closer to physiological conditions than when using platelet-poor or -rich plasma.

In the biosensor system, the magnitude of generated thrombin is expressed as intensity of electric current (Fig. 3). Although the presented curves are similar to conventional thrombin generation curves, they represent original raw data and not derivative curves. For signal interpretation, the curve progression of the sample is compared to that of normal plasma. Fig. 3B shows the correlation of a thrombin dilution series and the corresponding electric signal. The absence of lag times is explained by the immediate splitting of the substrate caused by the presence of thrombin from the very beginning of the reaction (in contrast to a plasma sample

in which thrombin has to be formed during the course of the coagulation process). The less thrombin that is present in the solution, the lower the peak height of the signal curve and the longer it takes to reach that point. Online supplemental Figs. 1–3 document a direct relationship between amount of thrombin present in the sample and height of the peak and time required to reach it. The integrated form of the Michaelis-Menten equation [$V_{\max} = p - \{K_m \times \ln(1 - p/s_0)\}$] describes a linear relationship of the maximum attainable velocity ($V_{\max}$) and the concentration of the product (p) of an enzyme-substrate reaction, wherein K_m is the Michaelis-Menten constant and s_0 the initial substrate concentration. In our system, there is a predicted linear dependence of the maximal slope (S-max) on thrombin concentration (shown in online Supplemental Fig. 1).

Besides thrombin, additional variables may affect the maximum in the current-vs-time curves, and all conditions influencing the shape of the curve after its peak are not fully elucidated at the moment. Therefore we consider parameters such as the curve peak and the area under the curve as yet not fully defined.

Our electrochemical procedure allows diverse predried reagents to be applied to the sensor strips. We have shown that we can activate the blood sample via extrinsic or intrinsic pathways (Fig. 4) and investigate the impact of exogenous APC (Fig. 5). Combining different types of sensor strips will allow to discriminate between dysfunctions caused by a defect in the intrinsic or extrinsic part of the coagulation cascade (Table 1). The form and the dimension of the curve progression depends on the trigger used. As expected, the intrinsically activated coagulation process starts later compared to the extrinsic one, in both plasma and whole blood. After activation by means of thromboplastin reagent as source of tissue factor, the TG curves of plasma and whole blood are comparable. Potential reasons the signal detected in whole blood is prolonged and less intense compared to that detected in plasma after intrinsic activation remain to be investigated. As demonstrated in prior work (18), the electrochemical signal negatively correlates with increasing hematocrit. The higher the amount of solid constituent in the same volume, the less plasma and therefore the lower the concentration of coagulation factors remains.

The usefulness of TG assays in the screening for FV G1691A (factor V Leiden) has already been discussed (19). With our method, we find a clear difference between normal samples and FVL samples, in platelet-poor plasma (Fig. 5A), already shown by Curvers et al. (20), as well as in whole blood (Fig. 5B). Interestingly, thrombin generation appears to be facilitated in whole blood compared to the respective platelet-poor plasma sample (compare the straight lines—FVL samples—and the dotted lines—normal samples—in Fig. 5B vs

5A). This is consistent with the observation made using the CAT method, in which platelet-rich plasma is significantly less sensitive to APC compared with platelet-poor plasma (8), and may be explained by the fact that coagulation factor Va on the surface of activated platelets is relatively protected from the action of APC (21). Finally, as demonstrated by the test setting to detect APC resistance by electrochemistry, we have shown the feasibility of predrying on the test strips reagents of interest such as APC.

The presented biosensor system provides a new method for recording thrombin generation. Several reagents can be predried on the test strips. There is the possibility to add reagents during the recording and so directly observe the effect on the formation of thrombin. This option may be a useful tool in the field of research, e.g., as an aid in studying procoagulant or anticoagulant activities, such as in the field of anticoagulant drug development. Further applications may be envisioned for daily routine analysis, e.g., monitoring of anticoagulants, monitoring of coagulation factor replacement treatment, or a general screening test for bleeding or thrombotic risks (or more specifically, e.g., for APC resistance). Moreover, the method allows assessment of TG as a point-of-care test, with a single drop of capillary whole blood in the medical practice, in the operating room, or for self-monitoring of a pharmaceutical treatment by the patient at home (18).

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