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A microfluidic anti-Factor Xa assay device for point-of-care monitoring of anticoagulation therapy†

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The development of new point-of-care coagulation assay devices is necessary due to the increasing number of patients requiring long-term anticoagulation in addition to the desire for appropriate, targeted anticoagulant therapy and a more rapid response to optimization of treatment. The majority of point-of-care devices currently available for hemostasis testing rely on clot-based endpoints which are variable, unreliable and limited to measuring only certain portions of the coagulation pathway. Here we present a novel fluorescence-based anti-Factor Xa (FXa) microfluidic assay device for monitoring the effect of anticoagulant therapy at the point-of-care. The device is a disposable, laminated polymer microfluidic strip fabricated from a combination of hydrophobic and hydrophilic cyclic polyolefins to allow reagent deposition in addition to effective capillary fill. Zeonor was the polymer of choice resulting in low background fluorescence (208.5 AU), suitable contact angles (17.5° ± 0.9°) and capillary fill times (20.3 ± 2.1 s). The device was capable of measuring unfractionated heparin and tinzaparin from 0 – 0.8 U/ml and enoxaparin from 0 – 0.6 U/ml with CVs < 10%. A linear correlation was observed between the device and the fluorescent assay in the plate for plasma samples spiked with UFH, with an R² value of 0.99, while correlations with tinzaparin and enoxaparin resulted in sigmoidal responses (R² = 0.99). Plasma samples containing UFH resulted in a linear correlation between the device and a standard chromogenic assay with an R² value of 0.98, with both LMWHs resulting in sigmoidal relationships (R² = 0.99).

Introduction

Arterial and venous thromboembolism remain two of the most frequent causes of morbidity and mortality worldwide. Blood clotting disorders such as these have long been treated using anticoagulant drugs such as warfarin and heparin, with newer, more predictable drugs such as factor Xa (FXa) and thrombin inhibitors now available. The administration of anticoagulant therapy is carefully monitored due to the potential adverse effects associated with over- or under-dosing. Traditional tests used for monitoring warfarin and heparin include clot-based assays such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests. These assays were traditionally executed in the central diagnostic laboratory on automated coagulation analysers, but have since been adapted to small, benchtop analysers, as well as handheld monitors for use at the point-of-care. Examples of such devices include the HemoSense INRatio monitor (Hemosense Inc., USA) for PT/INR home-testing, as well as the i-STAT (Abbott, USA) and Hemochron® analysers (ITC, USA) that can execute a range of clotting assays at the bench or bedside such as the PT, aPTT or ACT (activate clotting time).

Traditional anticoagulant drugs such as heparin have proven extremely effective. However, unfractionated heparin (UFH) is derived from animal sources such as porcine intestine and is highly variable in its molecular weight. This has unpredictable effects in its anticoagulant properties and so it must be closely monitored using clot-based assays such as aPTT and ACT. New anticoagulant drugs such as low molecular weight heparins (LMWHs) have defined pharmacokinetics and pharmacodynamics. Such drugs preferably target FXa rather than thrombin (FIIa) in the coagulation cascade, but traditional clotting assays are based on the measurement of thrombin formation and are not suitable for monitoring these new drugs. Other assays such as the anti-Factor Xa (anti-FXa) assay have been developed to measure the effect of such drugs. The anti-FXa assay operates via the addition of exogenous FXa to a plasma sample from a patient treated with an anticoagulant drug. Plasma contains antithrombin (AT) which binds to coagulation factors such as thrombin and FXa, so inhibiting their procoagulant function. However, anticoagulant drugs such as heparin bind to AT and significantly increase binding affinity for both thrombin and FXa. In addition, the high molecular weight of UFH stabilizes a ternary complex of AT, thrombin and heparin. Thus, in the presence of these anticoagulant drugs, the availability of free thrombin and FXa is significantly reduced in a dose-dependent manner, with a significant impact on thrombin generation. However, the LMWHs do not form stable ternary structures with AT and thrombin and so their antithrombin activity is substantially reduced, while their anti-FXa activity remains significant. Having been complexed with AT and anticoagulant drug, the concentration of free FXa is reduced and this can be titrated using a suitable substrate which is selectively cleaved by the serine protease activity of FXa. Pentapeptide substrates with chromophores for colorimetric assays are in widespread use. Unfortunately, absorbance-based assays are more prone to interference from blood and plasma. Recently, fluorogenic substrates have been employed as fluorescent labels which are inherently more sensitive than colorimetric substrates,
making these suitable labels in miniaturized diagnostic devices.\textsuperscript{9,10}

While clot-based assays for PT, aPTT and ACT have been developed for the point-of-care, no such devices exist for the performance of anti-FXa assays. With the increasing use of LMWHs, as well as the development of other drugs such as direct anti-Xa inhibitors (e.g. Rivaroxaban and Apixaban), the demand for such screening assays will increase. In addition, there is a major push for the screening of all individuals presenting upon emergency to be screened for their thrombotic risk profile, which is driving demand for development of point-of-care technologies in this area.\textsuperscript{11,12}

Current advances in point-of-care devices are often the result of microfluidic and Microsystems technologies which aim to deliver rapid results, enhanced sensitivity and specificity on low-cost, portable miniaturized devices.\textsuperscript{13,14} The development of a microfluidic device that uses optical detection such as fluorescence or absorbance requires the careful selection of device substrate materials. Glass has always been favored for optical applications due to its excellent clarity. However, its brittle nature results in processing and handling difficulties.\textsuperscript{15} Other inorganic materials such as silicon and quartz are not ideal for disposable microfluidics due to high material and manufacturing costs.\textsuperscript{16} The use of polymer-based microfluidics has gained in popularity over the last few years with substrates such as cyclic polyolefins (COPs) becoming more widely used in microfabrication. COPs offer many advantages for application to point-of-care devices such as low autofluorescence, high UV transmission, high temperature resistance and chemical inertness.\textsuperscript{16,17} In addition COPs can be modified to create hydrophilic surfaces from their native hydrophobic state which aids in fluid flow and control, without the incorporation of complex pumping systems, which is an important parameter in the design of point-of-care devices.

A fluorogenic anti-FXa assay was previously developed in our laboratory for monitoring heparin therapy in both human calibration plasmas and patient plasmas.\textsuperscript{9,18} In this paper we present a miniaturized, disposable device incorporating the fluorogenic anti-FXa assay. Data is presented showing that it can be used effectively to monitor heparin anticoagulation (UFH, enoxaparin and tinzaparin) in plasma samples, hence its suitability for application to point-of-care testing.

**Experimental**

**Reagents**

Water (ACS reagent) and HEPES (minimum 99.5% titration) were purchased from Sigma-Aldrich (Dublin, Ireland). Filtered HEPES was prepared at a concentration of 0.01 mM (pH 7.4). A 100 mM filtered stock solution of CaCl\textsubscript{2} from Fluka BioChemika (Buchs, Switzerland) was prepared from a 1 M CaCl\textsubscript{2} solution.

The fluorogenic substrate methylsulfonyl-D-cyclohexylalanylglycyl-arginine-7-amino-4-methylcoumarin acetate (Pefauflor™ FXa) was purchased from Pentapharm (Basel, Switzerland). It was reconstituted in 1 ml of water having a stock concentration of 10 mM, aliquoted, covered with aluminum foil to protect from exposure to light, and stored at -20 °C. Dilutions from 10 mM stock solutions were freshly prepared with 0.01 mM HEPES when required. Purified human FXa (serine endopeptidase; code number: EC 3.4.21.6) was obtained from HYPHEN BioMed (Neuville-Sur-Oise, France) and was reconstituted in 500 µl of PCR grade water to give a stock concentration of 4.4 M, with subsequent dilutions made with 10 mM HEPES buffer. Unfractionated heparin (UFH) (sodium salt of heparin derived from bovine intestinal mucosa, H0777) was sourced from Sigma-Aldrich (Dublin, Ireland), Enoxaparin (Clexane®) and Tinzaparin (Innohep®) were obtained from Sanofi-Aventis (Paris, France) and LEO Pharma (Ballerup, Denmark) respectively. Human pooled plasma was purchased from Helena Biosciences Europe (Tynemouth, UK). Lyophilized plasma was reconstituted in 1 ml of water and left to stabilise for at least 20 min at room temperature prior to use.

Rolls of 188 µm thick cyclic polyolefin polymer (Zeonor®) were purchased from IBIDI GmbH (Munich, Germany). ARcare® 92712 50 µm double sided pressure sensitive adhesive (PSA), BOPP-HY10 and HY10-coated Zeonor were purchased from Adhesives Research (Limerick, Ireland). Sheets of 188 µm Zeonor® was treated with a hydrophilic coating from Hydromer Inc. (NJ, USA). The Hydromer Anti-fog Coating 7-TS-13 is a proprietary, heat stable, non-yellowing, hydrophilic polymer coating. It is colourless, but appears clear to hazy when coated, it has a pH of 9.0-10.0 and a specific gravity of 0.805-0.865 g/ml at 25°C. Strip materials were cut using a Graphite Vinyl Cutter, Model CE5000-40-CRP from Graphitec GB Limited (Wrexham, UK). Contact angle measurements were carried out using an FTA 200 analyser from First Ten Angstroms, Inc. (Virginia, USA).

All fluorescent measurements were carried out at 37°C using an Olympus IX81 motorised fluorescence microscope sourced from Olympus Europa GmbH (Hamburg, Germany) housed within an incubation chamber with an attached Hamamatsu Orca ER digital camera, Model C4742-80-12AG from Hamamatsu Photonics (Hertfordshire, UK). Fluorescence was monitored according to the following settings: magnification x10; excitation at 342 nm and emission at 440 nm; exposure time of 20 ms. All values of fluorescence are reported as arbitrary fluorescence units (AU). All measurements were analyzed using the CellR realtime imaging software from Mason Technology (Dublin, Ireland) with subsequent data exportation into Excel, SigmaPlot 8.0, and SPSS 17.0 for analysis.

**Microfluidic assay and device**

For final strip assembly, the hydrophobic Zeonor lid (75 mm x 25 mm) was bonded to a PSA layer cut with a channel of 50 mm long x 2 mm wide, giving a total channel volume of 10 µl. The volume required to fill the channel was calculated as 50 x 2 x 0.05 mm to give 5 µl of sample. However a volume of 10 µl was selected so as to maintain a reservoir of sample at the channel inlet. Chips were monitored for 5 minutes at 37°C, hence this surplus prevented drying of the sample within the channel. The Zeonor lid was cut with an elliptical sample inlet of 4 mm diameter and a rectangular outlet of 4 x 1.5 mm to allow air to escape from inside the channel. 2 µl of Pefauflor™ FXa fluorogenic substrate was deposited with a pipette within the channel as four 0.5 µl droplets onto the hydrophobic Zeonor at a distance of 1.5 mm from the outlet (Fig. 1). Channels with deposited reagents were dried overnight in a glass desiccator with silica at 19°C and 10% RH (relative humidity). After drying, the
strips were pressure laminated and sealed with a hydrophilic Zeonor base.

All measurements for the fluorogenic anti-FXa assay were carried out in reconstituted citrated human pooled plasma. 4 µl of 0.26 µM FXa were incubated with 6 µl of re-calciﬁed plasma (44 µl heparinised plasma + 6 µl 100 mM CaCl₂) for 10 seconds and 10 µl of this FXa/plasma mixture was immediately applied to the inlet of the assay strip. Stock concentrations of anticoagulants were as follows: 20 KU tinzaparin, 15 KU enoxaparin, and 100 KU UFH. Stocks were further diluted (if stock was liquid) or made up to (if stock was solid) a concentration of 100 U/ml with PCR grade water. Plasma samples were then spiked with the required volume of 100 U/ml of anticoagulant to generate the pharmacologically relevant concentrations from 0 up to 0.8 U/ml.

Detailed experimental protocols on the ﬂuorogenic and chromogenic assay correlations can be found in the Supplementary Experimental Section S-1. In brief, absorbance and fluorescence measurements were performed in an Infinite M200 spectrophotometric microplate reader (Tecan Group Ltd, Männedorf, Switzerland). Plasma samples were spiked with pharmacologically relevant concentrations (0–0.8 U/ml) of therapeutic anticoagulants. The ﬂuorogenic assay was performed as previously described. Each well contained 6 µl of CaCl₂, 44 µl of spiked pooled plasma, and 50 µl of FXa. The reaction was started by adding 50 µl of Pefafluor FXa ﬂuorogenic substrate. The Biophen® Heparin chromogenic assay from Hyphen BioMed (Neuville-Sur-Oise, France) was carried out according to the manufacturer’s instructions as follows: each well contained 50 µl of spiked pooled plasma and 50 µl of antithrombin (AT). To this, 50 µl of FXa was added. The reaction was started by adding 50 µl of FXa specific chromogenic substrate.

For the development of point-of-care microﬂuidic assay devices, polymer-based materials are by far the most widely used due to the low cost of the materials, the range of simple fabrication methodologies available, their inert physical characteristics and good compatibility with biological materials. There are a range of popular polymer materials available such as polycarbonate (PC), polyethylene terephthalate (PET) and polymethylmethacrylate (PMMA). However, such materials do suffer from some disadvantages. For sensitive assays based on ﬂuorescence detection, their high background ﬂuorescence is a problem. An alternative group of polymers with low inherent ﬂuorescence are the cyclic polyolefins. These are now ﬁnding widespread use in bioassays in which sensitive fluorescence-based methods are used. These polymers like many others suffer from signiﬁcant hydrophobicity and so can be challenging when used with liquid biological samples such as blood where capillary ﬂow is required. A range of surface treatments and modiﬁcations have been developed to improve the hydrophilicity of these materials and this has made them viable materials for biodevice development.

A range of transparent polymer materials were initially screened for their background ﬂuorescence characteristics when excited at 342 nm. These were PMMA, PET, Zeonor® and Topas, the latter two being cyclic olein polymers. Among these, Zeonor® was found to have the lowest ﬂuorescence (data not shown). However, the hydrophobicity of the Zeonor ﬁlms was still problematic, preventing good capillary ﬂow. A number of cyclic polyolefins with various hydrophilic surface coatings were analyzed for their auto-ﬂuorescence properties, as well as their water contact angle measurements and capillary ﬁll characteristics when assembled into a simple capillary channel (Fig. 2). While both of the HY10-modiﬁed ﬁlms had excellent contact angles and capillary ﬁll times (7.6° ± 0.7° and 23 ± 1 s, respectively) the HY10 surface modiﬁcation contributed signiﬁcantly to the background ﬂuorescence (640.9 ± 2.3 AU, where maximum ﬂuorescence = 4000 AU). Unmodiﬁed Zeonor exhibited low ﬂuorescence as is well established. However, it showed high contact angles in excess of 100° and capillary ﬁll times were not recordable as liquid could not ﬂow along the channel, making it unsuitable. The Hydromer-modiﬁed Zeonor exhibited comparable ﬂuorescence with unmodiﬁed Zeonor of 208.5 AU, as well as having excellent contact angles (17.5° ± 0.9°) and capillary ﬁll times (20.3 ± 2.1 s).

In circumstances where the deposition of small volumes of aqueous-based reagents is required onto such polymeric surfaces, hydrophilic surfaces result in the deposited material spreading in an unpredictable manner across the surface due to its good wetting properties. Thus, a hybrid hydrophobic/hydrophilic design was proposed which would possess a hydrophobic layer for reagent deposition and a hydrophilic layer to induce capillary flow. Devices assembled using this hybrid approach were composed of materials with ﬁll times of 20.3 ± 2.1 s, contact angles of 17.5° ± 0.9° s and low background ﬂuorescence levels of 208.5 ± 0.1 AU which were intermediate to either fully hydrophobic or fully hydrophilic devices. Thus, further device development was based on Zeonor and Zeonor modiﬁed with Hydromer coating. The ﬁnal assay device conﬁguration was a three layer laminate strip comprising a 188 µm thick hydrophilic Zeonor base to induce capillary ﬂow, a 50 µm PSA spacer with

Fig. 1. Schematic and photograph of the microﬂuidic anti-FXa assay device. Strips were assembled from a hydrophobic Zeonor lid containing an inlet and outlet. The single straight channel was cut from PSA which seals the lid and base layers. The base layer was hydrophilic zeonor to allow for capillary ﬁll of the strip. Fluorogenic FXa substrate was deposited onto the hydrophobic lid prior to assembly.

Results and discussion
Strip materials characterization and selection

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channel and a 188 µm hydrophobic Zeonor lid for reagent deposition.

**Fig. 2.** Evaluation of device materials in terms of their auto-fluorescence, capillary fill times in assembled strip, and water contact angles (n=3).

**Assay optimization**

Using a plate-based fluorogenic anti-FXa assay as a guideline, the assay was transferred to and optimized on the strip format. FXa and Pefafluor™ FXa fluorogenic substrate were titrated within the range of 0.06-0.46 µM and 30-180 µM, respectively. Initial titrations were performed on chip with FXa and Pefafluor™ FXa dried onto the Zeonor surface. **Fig. 3** shows typical fluorescence response profiles of the strip to different concentrations of Pefafluor™ FXa substrate at a FXa concentration of 0.26 µM. This illustrates the conversion of fluorogenic substrate to fluorescent product which is limited by substrate concentration below 120 µM. After approx. 60 s, all substrate has been converted. It can also be observed that the maximum fluorescence signal that could be achieved with the available instrumentation was approximately 4000 AU which was achieved with substrate concentrations in excess of 60 µM. To ensure that substrate limitation did not result, a concentration of 150 µM was selected.

Titrations of FXa concentration were performed, maintaining Pefafluor™ concentration at 150 µM and varying FXa concentration from 0.06 to 0.46 µM at 0, 0.5 and 1 µ/ml heparin (**Fig. 4**). The effect of heparin on the response of each titration was analyzed and regression analysis was used to determine the optimal concentration of FXa. Regression analysis for 0.06 µM and 0.12 µM FXa returned R² values of 0.99. However, a wider signal range and a higher value at 0 U/ml was achieved at 0.26 µM with an R² value of 0.99. At higher concentrations of 0.36 µM and 0.46 µM FXa, regression analysis returned lower R² values of 0.93 and 0.87, respectively. Final assay concentrations were thus optimized at 150 µM fluorogenic substrate and 0.26 µM FXa and were tested using heparinised plasma samples at 0.25 U/ml intervals from 0 to 0.8 U/ml. Good separation was observed between heparin concentrations. The purpose of this experiment was to observe the impact of the FXa concentration across a suitable range of anticoagulant concentrations to ensure adequate discrimination across these concentrations. The slope for 0.06 µM is significantly lower than other concentrations due to the enzyme kinetics of the assay. At 0.06 µM after 60 s, the assay has not gone to completion and all available substrate has not been converted. However, at 0.12 µM, this has occurred. This is critical for obtaining the maximum zero value and maximum slope from the assay. The errors observed at 0 and 1 U/ml are naturally lower, as there has either been none or complete titration of the FXa with heparin, while at 0.5 U/ml, the level of titration is more sensitive to experimental variation.

**Fig. 3:** Typical fluorescence profiles of the anti-Xa assay with 0.26 µM FXa and concentrations of Pefafluor™ FXa from 30 to 180 µM (n=3).

**Fig. 4:** Fluorescence responses at 60 s for 0.06 to 0.46 µM FXa and 150 µM fluorogenic substrate at 0, 0.5 and 1 U/ml UFH (n=3).

Another important feature of assay and device development was sample volume. The device required 10 µl of plasma to execute a measurement, which is in line with point-of-care devices such as the CoaguCheck (Roche Diagnostics, UK), yet significantly lower than the 50 µl sample volume required to perform a test on the Hemochron® systems or the 20 µl sample volume required for application to the i-STAT analysers (Abbott, USA). The fast turnaround time of 60 seconds is also a significant advantage of the anti-FXa fluorogenic point-of-care device.
Optimization of substrate deposition

One of the most challenging aspects of the development of miniaturized diagnostic devices remains the effective deposition and subsequent resolubilisation of the assay reagents within the microfluidic device. Some aspects of this relate to the process used for deposition and others to the material being deposited. These factors can have a significant impact on assay performance and reproducibility. In the current assay device, four x 0.5 µl spots of the fluorogenic substrate were deposited in the microfluidic channel close to the measurement chamber. While automated methods of deposition are generally required for mass production to achieve control of volume and positional accuracy, it was found here that manual pipetting achieved reliable and reproducible results with spot diameters of 877 ± 51 µm with CVs of 6% (n=10), compared to inkjet printed spot diameters of 1094 ± 155 µm with CVs of 14 % (n=10). Further discussion on the optimization of substrate deposition can be found in the supplemental section S-2 Results and discussion.

Anticoagulant calibrations

The fluorescence responses of the optimized assay configuration were tested over a range of UFH concentrations. Fig. S-3 illustrates the typical fluorescent responses seen from the assay device (n=3). It can be seen that the fluorescence responses demonstrated inversely proportional dose-dependent rates of residual FXa enzymatic activity, with decreasing rates of product formation at higher drug concentrations, illustrating the anti-FXa activity of the AT/UFH complex formed. At lower drug concentrations, the responses appeared to show some deviation from linearity, with an initial upward trend, suggesting enzyme activity was increasing over this period. After approximately 43 s and 55 s, respectively, 0 and 0.2 U/ml heparin curves appeared to plateau. This is in part due to the reaching of the upper signal range achievable with the instrumental set up, but may also have a contribution from substrate limitation. Dosages above 0.6 U/ml had significantly titrated out all of the available FXa, with 1 U/ml showing little change over background levels.

Various methods can be employed to analyze the responses from these types of assays, including rates of change and responses at fixed time points. The log of the fluorescence responses at 30, 60 and 90 s were analyzed (Fig. S-1). Fluorescence at 30 and 90 s returned R² values of 0.99 and 0.93, with slopes of -0.915 and -1.11, respectively. However, the regression at 60 s yielded an R² of 0.97 and a slope of -1.12. Assay measurements performed at 60 s were shown to be capable of measuring UFH in the range of 0 to 0.8 U/ml with intra-assay CVs of 15% (n=3).

Two LMWH anticoagulants (tinzaparin and enoxaparin) were also tested on the anti-FXa microfluidic device and were analyzed along with UFH (Fig. 6). Linear regression analysis with enoxaparin returned an R² value of 0.98 with a linear correlation of \( y = -0.945x + 3.659 \), while analysis of tinzaparin yielded an R² value of 0.96 with a linear correlation of \( y = -0.789x + 3.637 \). Statistical analysis was performed on the logarithmically transformed datasets to assess intra-assay variability. When tested with enoxaparin-spiked plasmas a statistically sensitive range of 0 to 0.6 U/ml was achieved with CVs of 11%, while the dynamic range was 0.8 U/ml for plasma samples containing tinzaparin, with CVs of <12%.

Correlation of anti-Xa device with standard assays

For validation purposes, the microfluidic assay device was correlated with both a fluorogenic microtitre plate-based anti-FXa assay as previously described by Harris et al. and with the commercial Biophen® chromogenic assay from Hyphen BioMed (Neuville-Sur-Oise, France) (Fig. 7). A linear correlation was
observed between the device and the fluorescent assay in the plate for plasma samples spiked with UFH, with an excellent $R^2$ value of 0.99 (Fig. 7A). However, correlations of the device and the fluorogenic plate-based assay with tinzaparin and enoxaparin (Fig. 7C, 7E) resulted in sigmoidal correlations ($R^2 = 0.99$). In Fig. 7C the point at 0.4 U/ml tinzaparin resulted in a higher fluorescence response for both the assay on the device and the assay in the plate resulting in a correlation with just two points at 0 U/ml and 0.2 U/ml.

Correlations similar to that seen between with the fluorogenic plate-based assay were also observed with the chromogenic assay. UFH plasma samples again resulted in a linear correlation between the device and the chromogenic assay with an $R^2$ value of 0.98 (Fig. 7B). For both LMWHs tested in the chromogenic assay, the correlations with the device also had a sigmoidal relationship ($R^2 = 0.99$) (Fig. 7D, 7F). Although the reason for this is not clear, the linear correlations for UFH suggest a combination of both mechanistic factors relating to the pharmacokinetics of the drugs and their interplay with the kinetics of the microfluidic assay device. Nonetheless, correlations between the microfluidic device and both chromogenic and fluorogenic assays were achieved.

A point-of-care device based on the anti-Xa assay is not currently available so comparisons on levels of sensitivity and precision for this device can really only be drawn with clot-based devices for heparin measurement currently on the market. The aPTT is recommended for monitoring heparin therapy. However, there are serious limitations that accompany this assay in terms of the measurement range and the inter-laboratory variability. For example, clinically and statistically significant differences were observed between a point-of-care aPTT assay and a laboratory-based aPTT assay used to monitor patients on heparin therapy. Despite its disadvantages, the aPTT remains the most reliable and readily available test for clinicians, who will continue to use it until the emergence of a more accurate diagnostic test.

While many analysers exist and have been researched in the literature, the Hemochron® systems are the most widely accepted and commonly used, particularly for the measurement of aPTT. The Hemochron® system for aPTT measurement reports linearity with heparin up to 1.5 U/ml, at intervals of 0.25 U/ml and CVs of <10%. The Cascade® aPTT system from Helena Biosciences reports linearity up to 0.5 U/ml with an $R^2$ of 0.98, with intra and intervariability at <3% for normal and abnormal samples. A correlation of 0.72 with the anti-Xa chromogenic assay is also reported on the company datasheet. In the current study we observed linearity up to 0.8 U/ml with the anti-Xa device for UFH, enoxaparin and tinzaparin, with concentrations tapering off at 1 U/ml.

When the aPTT is compared to other clotting tests for heparin poor correlations are often observed, due to the variable responsiveness of commercial aPTT reagents to heparin. In terms of comparative studies, one research group compared bedside aPTT with laboratory aPTT using patients on heparin, with correlations varying from 0.13 to 0.67. Chavez et al. compared the CoaguChek™ Pro DM with core laboratory facilities for CPB (cardiopulmonary bypass) patients that received heparin therapy prior to surgery and a linear correlation ($R^2 = 0.83$) between the two assays was achieved. These findings were corroborated to a certain degree by Ferring et al. who reported poor agreement with patients after surgery but better agreement with healthy volunteer and control patient samples. In their study the anti-Xa assay also provided the best correlation with heparin dosage compared to point-of-care aPTT and central laboratory aPTT. In our study linear correlations ($R^2$ of 0.99) between the device and both plate-based assays were observed with control plasma samples containing UFH. The prothrombinase-induced clotting time assay (PiCT) is a point-of-care clotting time assay, sensitive to inhibitors of both thrombin and Xa. Calatzis et al. compared the PiCT with both aPTT and anti-Xa chromogenic assays for a range of anticoagulants. A non-linear response was observed with patients on fondaparinux, a synthetic inhibitor of FXa, while an almost linear response was achieved with patients on UFH, dalteparin, and enoxaparin. When comparing our device to the established chromogenic assay with samples spiked with LMWHs, we also observed non-linear correlations. However, using a sigmoidal treatment, excellent correlations of 0.99 were observed. This non-linearity with LMWH has also been reported by Coppell et al. who demonstrated a loss of linearity at LMWH concentrations above 0.5 U/ml when comparing TEG with a conventional aPTT clotting assay.
Fig. 7: Correlations of the anti-Xa assay in the microfluidic device with the anti-FXa plate-based assay and the Biophen® chromogenic assay for UFH (a-b), tinzaparin (c-d), and enoxaparin (e-f) (n=3).

Conclusions

The ability to measure the concentration of both unfractionated and low molecular weight heparin anticoagulant drugs in plasma was demonstrated using a polymer microfluidic device with integrated anti-FXa assay. Using fluorescence as the method of detection, the anti-FXa assay was executed on-chip resulting in the calculation of the concentration of heparin in the plasma sample. Fluorescent measurement after 60 s demonstrated assay ranges from 0 to 0.6 U/ml for enoxaparin and 0 to 0.8 U/ml for UFH and tinzaparin. The assay platform, a low cost, disposable laminate chip, was shown to correlate well against both standard chromogenic and fluorogenic anti-FXa assays performed in microtitre plates. In addition to its potential as a point-of-care device for the anticoagulant therapies, it could also be used as a
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Notes and references

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†S-1 Experimental section

Fluorogenic and chromogenic assay correlations

Absorbance and fluorescence measurements were performed in an Infinite M200 spectrophotometric microplate reader (Tecan Group Ltd, Männedorf, Switzerland) equipped with a UV Xenon flashlamp. Flat, black-bottom 96-well polystyrol FluorNunc™ microplates from Thermo Fisher Scientific (Roskilde, Denmark) were used for fluorescence measurements. Flat, transparent 96-well Greiner® microplates from Greiner Bio-One (Gloucestershire, United Kingdom) were used for absorbance measurements. All measurements for the fluorescent anti-FXa assay and the chromogenic assay were carried out in reconstituted citrated human pooled plasma. Pooled commercial plasma samples were spiked with pharmacologically relevant concentrations (0–0.8 U/ml) of therapeutic anticoagulants including UFH, enoxaparin, and tinzaparin.

For the fluorogenic assay, FXa and Pefaffluor FXa fluorogenic substrate concentrations were previously optimized as 0.004 µM and 0.9 µM, respectively. Each well contained 6 µl of 100 mM CaCl₂, 44 µl of pooled plasma, and 50 µl of FXa. The reaction was started by adding 50 µl of Pefaffluor FXa fluorogenic substrate. Samples within wells were mixed with the aid of orbital shaking at 37 °C for 30 s. Immediately after shaking, fluorescence measurements were recorded at 37 °C for 60 min, with a 20 µs integration time. Fluorescence excitation was at 342 nm and emission was monitored at 440 nm, corresponding to the excitation/emission wavelengths of the 7-amino-4-methylcoumarin (AMC) fluorophore. All the measurements were carried out in triplicate.

The Biophen® Heparin chromogenic assay from Hyphen BioMed (Neuville-Sur-Oise, France) was carried out according to...
the manufacturer's instructions as follows: each well contained 50 µl of plasma and 50 µl of antithrombin (AT). To this, 50 µl of FXa was added. The reaction was started by adding 50 µl of FXa specific chromogenic substrate. Samples within wells were mixed within the spectrophotometer by orbital shaking at 37 °C for 30 s. Immediately after shaking, absorbance measurements were recorded at 37 °C for 60 min, at 10 s intervals. Absorbance was measured at 405 nm and all measurements were performed in triplicate.

S-2 Results and discussion
Optimization of substrate deposition

A range of buffers (10 mM HEPES, 0.01 mM HEPES, StabilCoat buffer, 2% Tween 20, 10% PEG 3400, 1% Triton X-100) were tested for their effect on the dissolution, deposition and drying characteristics of the fluorogenic substrate. The morphology of the dried substrate spots can be seen in Fig. S-1 (A-F). It can immediately be seen from Fig. S-1 (A-F) that the matrices based on HEPES, StabilCoat and PEG produced uniform spots. However, the mixtures containing surfactant showed spot spreading and irregularity. Both the 10 mM HEPES and StabilCoat buffers showed a classical coffee-ring or doughnut morphology due to the movement of material to the edge of the droplet during drying. The dried polymer could also be seen in the sample containing PEG. However, 0.01 mM HEPES buffer resulted in uniform spots with few defects. Horizontal spot diameters measured on average 845 µm ± 59 µm (n=10; CV = 6.9%) and vertical spot diameters measured 877 µm ± 51 µm (n=10; CV = 5.8%).

Based on these morphological characteristics and fluorescent assay responses, HEPES buffer was found to be the most suitable matrix for the fluorogenic substrate. The impact of HEPES concentration on the performance of the substrate was further assessed. Three concentrations of HEPES buffer were subsequently tested for optimal performance in the assay using plasma or plasma with 0.5 U/ml UFH, 0.26 µM FXa and 150 µM substrate in 0.01, 0.05, and 0.1 mM HEPES (Fig. S-2). HEPES at 0.01 mM was selected as the optimal concentration as it returned the maximum fluorescence signal at 0 U/ml heparin and showed the greatest potential signal range with the smallest errors (1,896 AU between 0 U/ml and 0.5 U/ml).

The effect of temperature on the drying of the fluorogenic substrate was also assessed. Fluorogenic substrate prepared in 0.01 mM HEPES was dried onto the microfluidic devices using a range of temperatures and humidities. The most reliable and reproducible method of drying as determined from the morphology of the deposited spots proved to be drying at room temperature, at 10% RH, in a glass desiccator, which was subsequently adopted for preparation of assay devices.

Anticoagulant calibrations

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Table S-1: Comparison of data analysis methodologies based on linear regression

<table>
<thead>
<tr>
<th>Time of fluorescence measurement (s)</th>
<th>Regression equation</th>
<th>R² value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>y = -0.915x + 3.427</td>
<td>0.99</td>
</tr>
<tr>
<td>60</td>
<td>y = -1.124x + 3.665</td>
<td>0.97</td>
</tr>
<tr>
<td>90</td>
<td>y = -1.110x + 3.728</td>
<td>0.93</td>
</tr>
</tbody>
</table>