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A novel microfluidic anti-factor Xa assay device for monitoring anticoagulant therapy at the point-of-care

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ABSTRACT

Millions of patients worldwide are receiving anticoagulant therapy to treat hypercoagulable diseases. While standard testing is still performed in the central laboratory, point-of-care (POC) diagnostics are being developed due to the increasing number of patients requiring long-term anticoagulation and with a need for more personalized and targeted therapy. Many POC devices on the market focus on clot measurement, a technique which is limited in terms of variability, highlighting the need for more reliable assays of anticoagulant status. The anti-Xa assay, a factor specific optical assay, was developed to measure the extent to which exogenous factor Xa (FXa) is inhibited by heparin-antithrombin complexes.

We have developed a novel microfluidic device and assay for monitoring the effect of heparin anticoagulant therapy at the point-of-care. The assay which was also developed in our institute is based on the anti-Xa assay principle but uses fluorescence as the method of detection. Our device is a disposable laminate microfluidic strip, fabricated from the cyclic polyolefin (COP), Zeonor®, which is extremely suitable for application to fluorescent device platforms. We present data on the execution of the anti-Xa assay in this microfluidic format, demonstrating that the assay can be used to measure heparin in human plasma samples from 0 to 0.8 U/ml, with average assay reproducibility of 8% and a rapid result obtained within 60 seconds. Results indicate that with further development, the fluorogenic anti-Xa assay and device could become a successful method for monitoring anticoagulant therapy.

Keywords: Anti-factor Xa; capillary fill; fluorescence; low molecular weight heparin; unfractionated heparin; zeonor.

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INTRODUCTION

Cardiovascular disease such as arterial and venous thromboembolism are significant contributors to global morbidity and mortality.[1] As a result antithrombotics, such as heparins, are widely used in the treatment and prevention of thromboembolic disorders.[2] With advances in the pharmaceutical industry, newer, more predictable drugs for treating cardiovascular disease are being developed, such as thrombin and FXa inhibitors. Alongside low molecular weight heparin (LMWH), these newer drugs have a more defined and predictable pharmacokinetic/pharmacodynamic profile and have a preference for FXa as a therapeutic target over thrombin.[3]

Traditionally clotting disorders were monitored using standard clot-based tests, which were executed in the central diagnostic laboratory on automated coagulometers.[4] Many of the standard clotting assays such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) have been translated into miniaturised POC format allowing for ease of testing, be it at the bench or bedside. For example, the lives of warfarin patients have been transformed with the development of the HemoSense INRatio monitor (HemoSense Inc., USA) for PT/INR home-testing.

Traditional testing principles do, however, remain limited in terms of poor predictive value, poor quality and accuracy. Insensitivity to certain anticoagulants is also another issue as they are often based on the measurement of thrombin formation.[5]

Peptide substrates specific for coagulation proteins were developed in the 1960s[6], one of which led to the development of a chromogenic anti-Factor Xa assay. Considered the 'gold standard' for monitoring heparin therapy, it is regularly performed in the central laboratory. However, currently there are no POC devices available on the market that use this technology.

POC technologies are being revolutionized by progress in the fields of microfabrication and microfluidics to achieve a low cost, easy to use, portable device that can generate rapid results. Polymer-based materials are often used in microfluidic device development due to their versatility in terms of application and manipulation but more importantly due to their low cost. The cyclic polyolefins (COPs) are a class of polymers employed due to their compatibility with biological materials, low autofluorescence and their suitability for surface modification amongst others.[7,8]

In our laboratory we have developed a low cost, disposable, miniaturised POC device that uses the anti-Xa assay principle in conjunction with a fluorescent measurement technique and is capable of measuring therapeutic concentrations (0 – 0.8 U/ml) of heparin in human plasma samples.

METHODOLOGY

1.1 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 $CaCl_2$ from Fluka BioChemika (Buchs, Switzerland) was prepared from a 1 M $CaCl_2$ solution.

The fluorogenic substrate methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Pefafluor™ FXa) was purchased from Pentapharm (Basel, Switzerland). It was reconstituted in PCR grade water, aliquoted, covered with aluminum foil to protect from exposure to light, and stored at -20 °C. 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 PCR grade water. Unfractionated heparin (UFH) (sodium salt of heparin derived from bovine intestinal mucosa, H0777) was sourced from Sigma-Aldrich (Dublin, Ireland). Human pooled plasma was purchased from Helena Biosciences Europe (Tyne and Wear, 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) and HY10-coated Zeonor was purchased from Adhesives Research (Limerick, Ireland). Sheets of 188 µm Zeonor® was treated with a hydrophilic coating from Hydromer Inc. (NJ, USA). Strip materials were cut using a Graphtec Vinyl Cutter, Model CE5000-40-CRP from Graphtec 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 fluorescent 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 ×10; 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 Cell`R realtime imaging software from Mason Technology (Dublin, Ireland) with subsequent data exportation into Excel, SigmaPlot 8.0, and SPSS 17.0 for analysis.

1.2 Microfluidic assay and device development

The microfluidic chip was developed in two stages. In the first stage the fluorogenic substrate was deposited within the microfluidic channel and the FXa was incubated externally with plasma prior to application. In the second stage of development, the FXa reagent was incorporated on chip to present a fully integrated device.

Stage 1 strip development

For stage 1 final strip assembly, the hydrophobic Zeonor lid was bonded to a PSA channel layer. The Zeonor lid was cut with an elliptical sample inlet and a rectangular outlet to allow air to escape from inside the channel. Pefafluor™ FXa fluorogenic substrate was deposited within the channel onto the hydrophobic Zeonor at a distance of 1.5 mm from the outlet (Figure 1). Channels with deposited reagents were dried for 2 hours in a glass desiccator with silica at 19°C and 10% RH. After drying, the strips were pressure laminated and sealed with a hydrophilic Zeonor base.

All measurements for the fluorogenic anti-Xa assay were carried out in reconstituted citrated human pooled plasma. FXa was incubated with re-calcified plasma for 10 seconds and 10 µl of this FXa/plasma mixture was immediately applied to the inlet of the assay strip. Plasma samples were spiked with pharmacologically relevant concentrations (0–0.8 U/ml) of UFH.

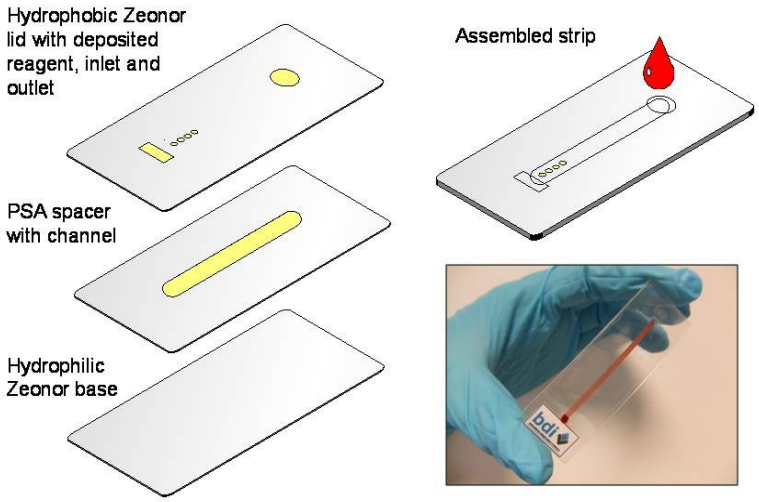

Figure 1. Schematic and photograph of the microfluidic anti-FXa assay device. Strips were assembled from a hydrophobic Zeonor lid containing an inlet and outlet. The single straight channel was cut from PSA which seals the lid and base layers. The base layer was hydrophilic Zeonor to allow for capillary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

Stage 2 strip development

For stage 2 final strip assembly, Zeonor and PSA were cut into multichannel sheets using the Graphtec Vinyl Cutter. The channel dimensions were identical to those in stage one. All 3 layers contained alignment marks for easy assembly.

The PSA layer was bonded to the Zeonor lid layer using the alignment marks and Pefafluor™ FXa fluorogenic substrate was deposited within each channel onto the hydrophobic Zeonor as in stage 1. The fully integrated device contained FXa integrated on chip compared to external FXa/plasma incubation in stage 1. Strips were dried for 2 hours in a glass desiccator with silica at 19°C and 10% RH. After drying, the sheets of PSA/Zeonor were bonded to the hydrophilic Zeonor base layer using pressure lamination.

Once the strip was assembled, re-calcified plasma was immediately applied to the inlet of the assay strip. Plasma samples were spiked with pharmacologically relevant concentrations (0–0.8 U/ml) of UFH.

RESULTS

1.3 Strip materials characterization and selection

Zeonor was the polymer of choice in developing the microfluidic chip due to its excellent optical properties for fluorescence measurements. Zeonor is highly hydrophobic and as this device works on the principle of capillary fill, modification of the hydrophobic Zeonor surface was investigated. A range of commercially available hydrophilic materials were tested in terms of autofluorescence, capillary fill, and contact angle (Table 1).

Table 1: Comparison of commercial hydrophilic surfaces in terms of fluorescence, capillary fill, contact angle and the variability associated with each measurement parameter (n=3).

	IBIDI Zeonor	HY10 Zeonor	Hydromer Zeonor
Fluorescence (AU)	202.3 ± 0.1	640.9 ± 2.3	208.5 ± 0.1
Capillary fill (s)	101 ± 11.5	23 ± 1	20.3 ± 2.1
Contact angle (°)	63.4 ± 2.8	7.6 ± 0.7	17.5 ± 0.9

While the HY10-modified Zeonor returned excellent capillary fill times (23 ± 1) and contact angles (7.6 ± 0.7), there was a significant contribution to the background fluorescence from the HY10 modification (640.9 ± 2.3). Zeonor modified with the IBIDI surface treatment showed fluorescence comparable with unmodified Zeonor (202.3 ± 0.1), but the wettability of the surface was poor (101s ± 11.5 (capillary fill); 63.4° ± 2.8 (contact angle)). The Hydromer treated Zeonor was finally selected owing to its low autofluorescence of 208.5 AU in the UV range of the spectrum. In addition to low autofluorescence, contact angles from this Hydromer treated Zeonor were 17.5° ± 0.9, resulting in reproducible capillary fill times of 20.3 s ± 2.1.

A hybrid hydrophobic/hydrophilic device combining unmodified Zeonor with Hydromer-coated Zeonor was designed allowing for reagent deposition onto the hydrophobic layer and a hydrophilic layer to induce capillary flow. The final assay configuration was a three layer laminate strip comprising a 188 µm thick hydrophilic Zeonor base, a 50 µm PSA spacer with channel and a 188 µm thick hydrophobic Zeonor lid.

1.4 Assay optimization and development

The anti-Xa assay operates via the addition of exogenous FXa to a heparinised plasma sample, which is incubated to allow the antithrombin (AT) in the sample to form a complex with the excess FXa and heparin. The formation of this complex allows AT to bind to thrombin and FXa more strongly, resulting in their inhibition. The concentration of free FXa can then be measured using a substrate that is selectively cleaved by the serine protease activity of FXa. Once cleaved, the peptide substrate will release a chromophore that can be detected colorimetrically.

We have developed a fluorogenic anti-Xa assay which can be executed on both of the microfluidic devices described above. The assay was optimized for each device.

For the chip in stage 1, FXa and Pefafluor FXa™ fluorogenic substrate were titrated within the range of 60-460 nM and 30-180 µM respectively. Figure 2 (a) shows the typical fluorescence profiles of a fixed concentration of FXa to varying concentrations of Pefafluor™ FXa substrate and Figure 2 (b) shows the fluorescence responses at 60 s for a range of FXa concentrations at a fixed concentration of Pefafluor™ FXa in plasma at concentrations of 0, 0.5, 1 U/ml UFH. The optimal fluorogenic substrate concentration was selected at a concentration that did not limit the conversion of the substrate to product. To select the optimal FXa concentration, the effect of heparin was analyzed using regression analysis. The optimal concentration of FXa was selected as it resulted in a wider signal range than other concentrations and an R^2 value of 0.99.

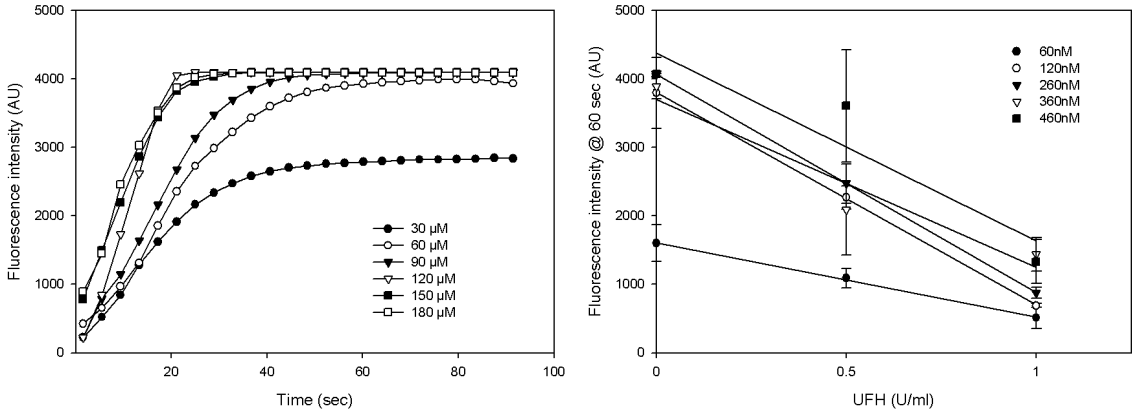

Figure 2. (a) Typical fluorescence profiles of the anti-Xa assay at a fixed concentration of FXa and varying concentrations of Pefafluor™ FXa; (b) Fluorescence responses at 60 s for a range of FXa concentrations and a fixed concentration of Pefafluor™ FXa at 0, 0.5, and 1 U/ml UFH.

For the chip in stage 2, FXa and Pefafluor FXa™ fluorogenic substrate were titrated within the range of 560-760 nM and 100-400 µM respectively. Figure 3 (a) shows the typical fluorescence profiles of a fixed concentration of FXa to varying concentrations of Pefafluor™ FXa substrate and Figure 3 (b) shows the fluorescence responses at 60 s for 560-760 nM FXa and a fixed concentration of Pefafluor™ FXa in plasma at concentrations of 0, 0.5, 1 U/ml UFH. The data was analyzed as for stage 1 and optimal concentrations were selected.

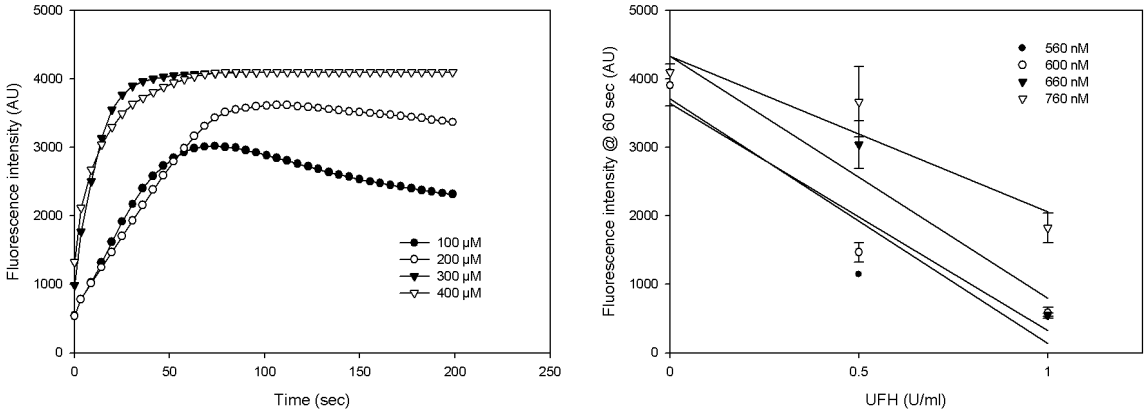

Figure 3. (a) Typical fluorescence profiles of the anti-Xa assay at a fixed concentration of FXa and varying concentrations of Pefafluor™ FXa; (b) Fluorescence responses at 60 s for a range of FXa concentrations and a fixed concentration of Pefafluor™ FXa at 0, 0.5, and 1 U/ml UFH (n=3).

1.5 Calibrations of UFH

The fluorescence responses of the optimized assay conditions for both chips were tested over a range of UFH concentrations. It can be seen from the fluorescence responses in Figure 4 (a) that the concentration of heparin is inversely proportional to the rate of fluorescence formation. As the concentration of drug increases, the rate of fluorescence product formation decreases. Figure 4 (b) outlines the dose response curve of human plasma spiked with UFH as tested in stage one of the anti-Xa device. Various methods were investigated for optimal data analysis and extraction but the fluorescence value at 60 sec was most appropriate returning an R^2 value of 0.95 ($y = -4675.4x + 3983.7$). Assay reproducibility was good with an average CV of 8.3%.

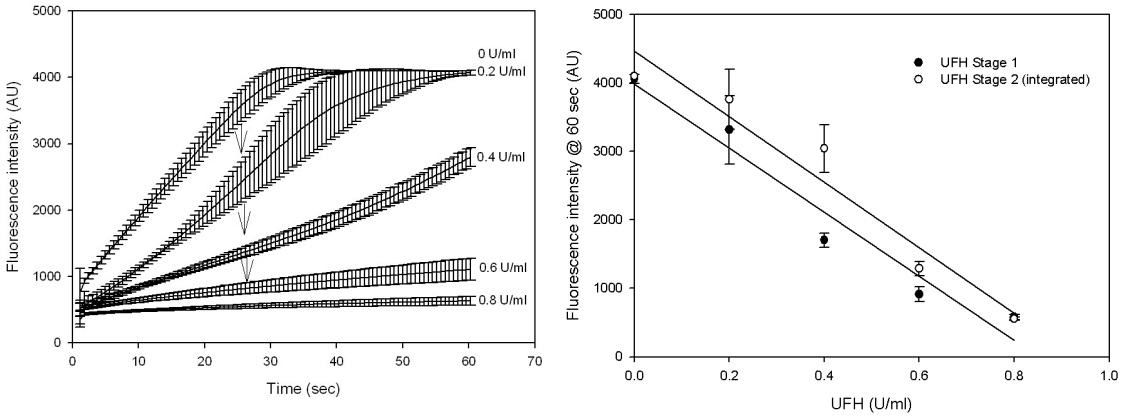

Figure 4. (a) Fluorescence response profiles of human plasmas in the anti-Xa assay device supplemented with concentrations of UFH from 0 – 0.8 U/ml (n=3); (b) Dose response curve of human plasma spiked with UFH from 0 – 0.8 U/ml in stage 1 and stage 2 (fully integrated) of the anti-Xa device (n=3).

The same analysis was performed with the fully integrated anti-Xa device. Human plasma samples spiked with UFH were tested and linear regression analysis was performed. The fluorescence value at 60 sec was the most appropriate analysis parameter and returned an R^2 value of 0.94 ($y = -3819x + 4457.8$) with an average CV of 7.1%.

DISCUSSION AND CONCLUSIONS

While coagulation monitoring devices occupy a large portion of the POC market, there is significant scope for the development and introduction of novel devices, which offer improvements over conventional clotting tests for anticoagulant monitoring. Peptide substrates specific for coagulation proteins such as thrombin and plasmin emerged in the 1960s[9,10] with the first anti-Xa chromogenic assay for heparin monitoring developed in 1976.[6] The chromogenic anti-FXa assay is now classed by central diagnostic laboratories as the 'gold standard' assay for measuring heparin anticoagulant therapy in plasma samples. While it reigns superior over older traditional clot-based assays, it has not yet been translated into a POC device.

The focus of our research was on the miniaturisation of a fluorogenic anti-FXa assay developed in our laboratory for monitoring heparin anticoagulation in cardiac patients and its incorporation into a microfluidic POC device. The device was designed to achieve a simple configuration without pumping systems or complicated fabrication methodologies. The three-layer laminate was comprised of 188 µm Zeonor which was easily manipulated in terms of manufacture with the Graphtec Vinyl Cutter, as opposed to more complex microfabrication technologies such as laser ablation, micromilling or hot embossing, which can be costly to operate and require skilled operators. Commercially available polymer materials such as polycarbonate (PC), polyethylene terephthalate (PET) and the cyclic polyolefin (COP) are popular candidates for the development of POC devices due to their low cost and suitability for biological applications.[11,12] COP polymers such as Zeonor, offer device development many advantages such as excellent optical clarity, high UV transmission, easy modification of its surface and suitability for disposable POC devices.[13] In this study Zeonor was the polymer of choice as it additionally resulted in low background fluorescence at our excitation wavelength of 342 nm.

The device presented here uses the simple principle of capillary force which minimizes the integration of complex, sophisticated, and costly instrumentation such as pumps or valves into the device. The concept of capillary force has long been used in lateral flow tests which are the most commercially successful POC platforms due to their simplicity.[14] The hydrophobicity of Zeonor is suitable for reagent deposition but is problematic in the development of a device based on capillary fill as it prevents flow. Three types of surface modification were analyzed on the device and the Hydromer-modified Zeonor with low water contact angles of 17.5° ± 0.9, produced the most reproducible capillary fill times of 20.3 s ± 2.1. As a result the device configuration was a joint hydrophilic/hydrophobic design to incorporate both the reagent deposition and capillary fill components of the device.

Human plasma samples containing therapeutic levels of UFH were tested on the assay and device in both stage 1 and stage 2 of the device development. We demonstrated that the stage 1 device was capable of measuring UFH in human plasma from 0 to 0.8 U/ml with average intra-assay CVs of 8.3%. The fully integrated device developed in stage 2 showed sensitivity in the therapeutic range (0 – 0.8 U/ml) with an average CV of 7.1%.

Comparisons on levels of sensitivity and precision can only be drawn with clot-based devices for heparin measurement currently on the market, as an optical POC system for anticoagulant monitoring is not currently available. While many analysers exist and have been researched in the literature, the Hemochron® systems are most widely accepted and commonly used. Hemochron® aPTT measurements report linearity with heparin up to 1.5 U/ml, at intervals of 0.25 U/ml and CVs of <14%. While the ACT is a clot-based POC assay used to monitor patients on heparin therapy it is performed when patients are undergoing cardio-pulmonary bypass (CPB) and are on high doses of heparin (>1 U/ml heparin) which is beyond the normal therapeutic range that is presented in the anti-FXa assay and device in the current study.

Another advantage of the system presented here is the small sample volume of 10 µl/20 µl required to execute a measurement and fast turnaround time of 60 seconds, which is in line with POC 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. With further development, the fluorogenic anti-Xa assay and device could prove successful for near-patient monitoring of antithrombotics with the knock-on effect of improving the health of patients on anticoagulant drugs.

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