



Analytical Methods

ARTICLE

A fully integrated microfluidic device for point-of-care monitoring of antithrombotics

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The simplicity and efficiency of point of care diagnostics have revolutionised patient care. Current methods for measuring hypercoagulability often require trained technicians, large blood volumes, and result in long turnaround times. Standard testing for hypercoagulable disorders is performed in the central laboratory using automated coagulation analysers. However the trend is moving towards the development and implementation of point of care testing, as a result of the ever increasing number of patients on antithrombotic therapy. We present a novel microfluidic device and assay for monitoring the effect of two anticoagulants, unfractionated heparin (UFH) and low molecular weight heparin (LMWH). The assay is based on the anti-Xa assay principle but uses fluorescence 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 both UFH and LMWH in human plasma samples from 0 to 1 U/mL, with a rapid result obtained within 30-60 seconds.

Introduction

Antithrombotics are commonly administered for the treatment of arterial and venous thromboembolism. Unfractionated heparins and low molecular weight heparins are some of the traditional antithrombotics used in the treatment and prevention of such thromboembolic disorders.¹ Low molecular weight heparin (LMWH) has a more defined and predictable pharmacokinetic or pharmacodynamic profile over unfractionated heparin (UFH) and it also prefers FXa as a therapeutic target over thrombin.²

Standard testing for coagulation disorders are usually based on the principle of clotting and are carried out on automated coagulometers.³ However with the advent of point of care (POC) diagnostics, many of the standard clotting assays such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) have been translated into miniaturised POC devices. Warfarin is commonly monitored using the HemoSense INRatio monitor (HemoSense Inc., USA) which was developed for determining the PT/INR of patients undergoing oral anticoagulation treatment. Testing for anticoagulant therapy is very diverse with numerous different tests available for assessing coagulation status, however diagnostics based on the traditional testing principles are

limited in terms of poor predictive value, poor quality and accuracy.⁴ As a result there is a drive for the standardization of current coagulation tests alongside a constant push for the development of a global test of haemostasis.⁵

The emergence of peptide substrates in the 1960s⁶, had a knock-on effect with the development of substrates specific for coagulation proteins. As a result, the chromogenic anti-factor Xa (anti-FXa) assay emerged as the new standard for monitoring LMWH therapy in the central laboratory. The anti-FXa assay works on the principle of 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 and inhibit thrombin and FXa more strongly. The concentration of free FXa can then be measured using a substrate that is selectively cleaved by the serine protease activity of FXa.⁷ Once cleaved, the peptide substrate will release a chromophore that can be detected colorimetrically.

As the field of microfluidics has advanced, POC technologies have also developed and improved. Methods in microfabrication and microfluidics have progressed so much in the last few years that POC devices can be fabricated at low cost, they are easy to use, portable and can generate rapid results.^{8,9} Silicon, glass, and polymers are often the substrates of choice in the manufacture of microfluidic devices as their chemical and physical properties are well characterized. Silicon and glass can pose issues due to costly fabrication processes, while polymers are low cost. Polymer-based materials are highly versatile in that they suit many modes of fabrication, are suitable for modification with surface chemistries and are also highly reproducible.¹⁰ In the current study we have used cyclic polyolefins (COPs) which belong to a class of polymers employed due to their compatibility with biological materials, low

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autofluorescence, long shelf life and suitability for surface modification amongst others.^{11,12} Multilayer alignment fabrication methodologies are commonly used to develop functional microdevices^{13,14}, as lamination of polymer layers offers greater versatility and low cost. Zeonor® was chosen for this study, in conjunction with PSA (pressure sensitive adhesive), as both are suitable materials for simple fabrication and lamination methodologies.¹⁵

In our study we have developed a low cost, disposable, miniaturised POC device. We have developed a three layered hybrid chip, comprised of native hydrophobic Zeonor®, treated hydrophilic Zeonor® and a PSA spacer that forms the channel.¹⁶ The assay is incorporated onto the chip and works on the basis of the anti-Xa principle outlined above. Using fluorescence as the method of detection, the assay is capable of measuring therapeutic concentrations of heparin in calibration plasmas from 0-1 U/mL and in plasma samples from patients on heparin therapy.

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 CaCl₂ from Fluka BioChemika (Buchs, Switzerland) was prepared from a 1 M CaCl₂ solution.

The fluorogenic substrate methylsulfonyl-D-cyclohexylalanyl-glycyl-arginine-7-amino-4-methylcoumarin acetate (Peflafluor™ 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. 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 4400 nM. Unfractionated heparin (UFH) (sodium salt of heparin derived from bovine intestinal mucosa, H0777) was sourced from Sigma-Aldrich (Dublin, Ireland). Low molecular weight heparin tinzaparin (Innohep®) was obtained from Leo Pharma (Ballerup, Denmark). 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). Conjugate pads (CP) Type 8301 p/n SMCONO1 were sourced from Pall Europe Ltd. (Portsmouth, England). 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 22.0 for analysis.

Microfluidic Assay and Device Development

Development of the microfluidic chip was divided into two stages. In the first stage the fluorogenic substrate was deposited within the microfluidic channel, while the FXa reagent was incubated externally with plasma prior to its application on-chip. The results of this study have been published previously.¹⁶ In the second stage of development, the aim was to develop a fully integrated device with all reagents incorporated on chip.

Integrated Strip Development

For the development of the integrated strip (Fig. 1), Zeonor® and PSA were cut into multichannel sheets (13 strips) using the Graphtec Vinyl Cutter (Fig. 2) from drawings designed using AutoCAD® 2007. The Zeonor® lid was cut with thirteen elliptical sample inlets of 4 mm diameter and rectangular outlets of 4 x 1.5 mm to allow air to escape from inside the channels. The PSA channels were 50 mm long x 2 mm wide, with an additional area cut across the channels at the inlet end, which measured 15 mm in length by 5 mm width (Fig. 2). All 3 layers contain alignment marks for easy assembly.

The CP was also cut to the same dimensions of 15 cm in length and 5 mm wide (Fig. 2). Each CP was dip coated in 200 µl of FXa and left to dry for 2 hours in a desiccator at 19°C and 10% RH. Dip coating of the conjugate pad took place in a "microdip tank vessel". A scalpel was used to cut the bulb and nozzle from a disposable pipette. The trough created from the bulb is used to contain the FXa. The pipette tip is placed over the needle, to provide a roller which attempts to mimic a standard dip tank coating procedure, while avoiding metal contamination from the needle. Both parts are disposed of after use to avoid cross contamination with future batches.

The PSA layer was bonded to the Zeonor® lid layer using the alignment marks and 2 µl of Peflafluor™ FXa fluorogenic substrate was deposited with a pipette within each channel as four 0.5 µl droplets onto the hydrophobic Zeonor® at a distance of 1.5 mm from the outlet. Sheets were dried for 2 hours in a glass desiccator with silica at 19°C and 10% RH. After drying, the CP was inserted into the cut rectangular area on the PSA and bonded to the hydrophilic Zeonor® base layer using pressure lamination.

Once the strip was assembled, 20 µl of re-calcified plasma (44 µl heparinized plasma + 6 µl 100 mM CaCl₂) was immediately applied to the inlet of the assay strip. Plasma samples were spiked with

pharmacologically relevant concentrations (0–1 U/mL) of UFH and LMWH.

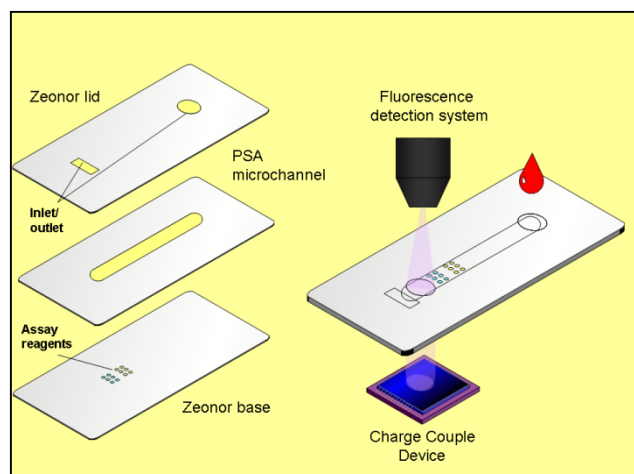


Fig. 1. Schematic of the microfluidic chip highlighting the hydrophobic Zeonor® lid layer (with inlet and outlet), the PSA microchannel layer and the hydrophilic Zeonor base layer with deposited reagents. The image to the right incorporates the microdevice with a miniaturised detection system.

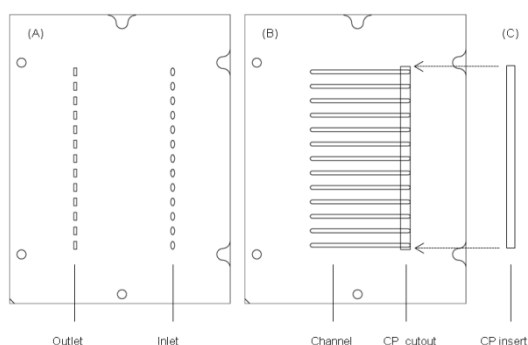


Fig. 2. Multichannel Zeonor® lid layer (A), PSA channel layer (B) and CP containing the FXa reagent (C). The PSA layer containing the CP in the inlet area, was sandwiched between the hydrophilic Zeonor® lid layer and a hydrophobic Zeonor® base layer.

Patient Samples

A small preliminary study using patient samples was carried out. Plasma samples from patients on UFH were kindly donated by Tomas Lindahl based in the Linköping University Hospital, Sweden. Ten plasma samples from patients on UFH were collected and tested on our microfluidic device platform. Correlations were performed with the assay standard, aPTT for UFH. Patient samples were thawed at 37 °C in a water bath for 5 min and inverted for 5 min before testing on the anti-FXa microfluidic device. All measurements were carried out in triplicate.

Results and Discussion

Strip materials characterisation and selection

Zeonor® was selected as the polymer of choice for microfluidic chip development due to excellent optical characteristics for fluorescence measurements. A range of surface modifications were tested to render the hydrophobic Zeonor® surface hydrophilic. This allowed for capillary fill of the microfluidic chip. A range of 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 (RFU)	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

The HY10 treatment was not selected owing to its significant contribution to background fluorescence (640.9 AU ± 2.3). Fluorescence readings from the IBIDI Zeonor® and the Hydromer Zeonor® were comparable, but the wettability of the IBIDI treatment was poor by comparison with contact angles of 63.4° ± 2.8. As a result the Hydromer treated Zeonor® was selected with a low autofluorescence (208.5 AU) in the UV spectrum, low contact angles (17.5° ± 0.9) and capillary fill times of 20.3s ± 2.1.

A hybrid microfluidic device was developed by combining unmodified Zeonor® with Hydromer-coated Zeonor®. This allowed for deposition of reagent onto the hydrophobic film, while the hydrophilic film served to induce capillary fill. The final assay configuration was a three layer laminate chip comprising a 188 µm thick hydrophilic base, a 50 µm PSA spacer with a channel, into which the conjugate pad was inserted and a 188 µm thick hydrophobic Zeonor® lid.

Assay Optimisation and Development

For the microfluidic chip developed, FXa and Pefalfluor FXa™ fluorogenic substrate were titrated within the range of 560–760 nM and 100–400 µM respectively. Fig. 3 (a) shows the typical fluorescence profiles of a fixed concentration of 600nM FXa to varying concentrations of Pefalfluor™ FXa substrate and Fig. 3 (b) shows the fluorescence responses at 60 s for 560–700 nM FXa and a fixed concentration of 300 µM Pefalfluor™ FXa in plasma at concentrations of 0, 0.5 and 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, which was 660nM returning an R² of 0.98.

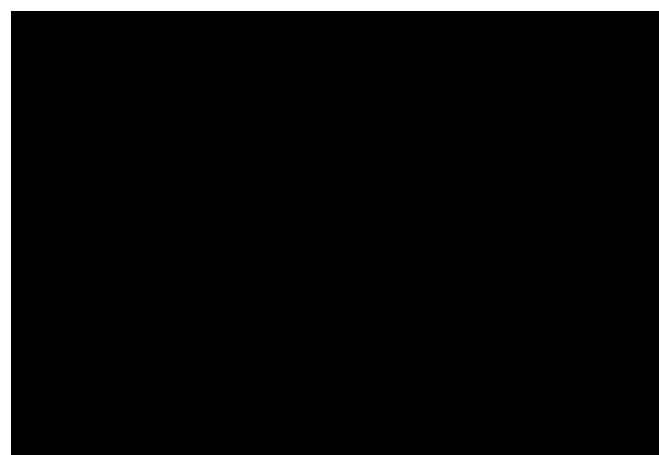
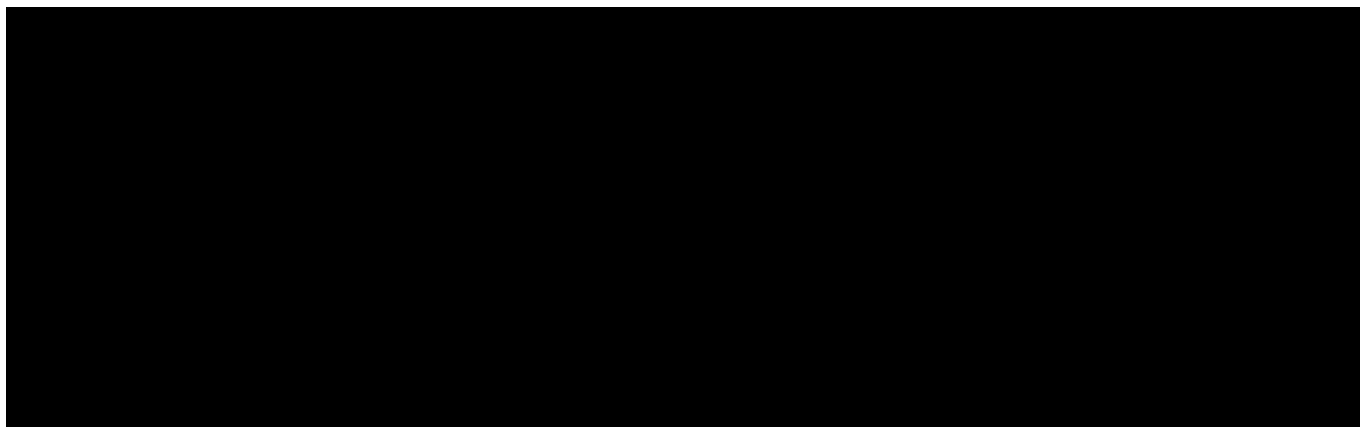


Fig. 3. (a) Typical profiles of the anti-Xa assay at a fixed concentration of 660 nM FXa and varying concentrations of Pefafleur™ FXa. (b) Fluorescence responses at 30 s for a range of FXa concentrations and a fixed concentration of 300 μM Pefafleur™ FXa at 0, 0.5, and 1 U/mL UFH (n=3).

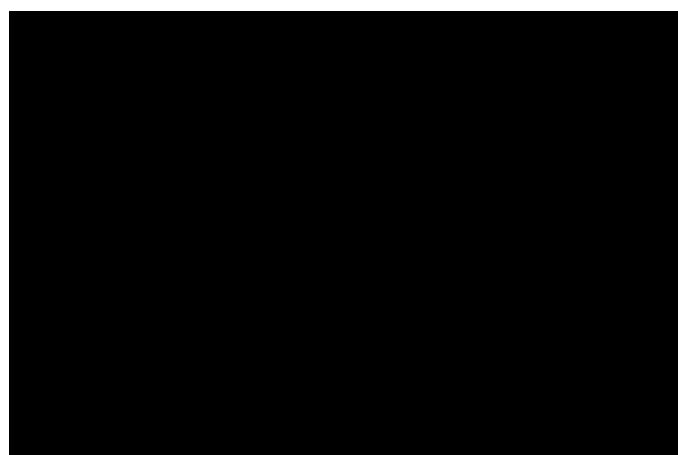


Fig. 4: Linear regression analysis of fluorescent responses at 30, 60 and 90 seconds for (a) UFH and (b) LMWH. The fluorescence value @ 30 seconds was selected as the most appropriate parameter for measuring UFH, while the fluorescence value at 60 seconds was the most appropriate measurement parameter for LMWH.

To select the optimal FXa concentration, the effect of heparin was analyzed using regression analysis (Fig. 4). The optimal concentration of FXa was selected that resulted in a wider signal range than other concentrations, small error and an R^2 value close to 0.99. The optimal concentrations were selected as 300 μM Pefafleur™ FXa substrate and 660 nM FXa for the final assay development of the UFH assay (Fig. 4a). The FXa and Pefafleur™ FXa fluorogenic substrate concentrations for the LMWH assay were optimized as previously using regression analysis (Fig. 4b). Final concentrations were selected as 650 nM FXa and 600 μM Pefafleur™ FXa fluorogenic substrate.

The fluorescence responses of the optimized assay conditions were tested over a range of UFH and LMWH concentration. 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. Fig. 5 outlines the dose response curves of human plasma spiked with UFH and LMWH in the anti-Xa device. Various methods were investigated for optimal data analysis and extraction, but the fluorescence value at 30 sec was most appropriate for UFH returning an R^2 value of 0.98 ($y = -4309.3x + 4061.1$) (Pearson = 0.99). The fluorescence value at 60 sec was selected as the most appropriate value for LMWH giving an R^2 value of 0.97 ($y = -3630x + 4053.8$) (Pearson = 0.99). Assay reproducibility was good with average CVs of <12% with one exception at 0.25 U/mL for both the UFH and LMWH assays. Variability at 0.25 U/mL was 35% for UFH and 19% for LMWH assay. Statistical analysis returned significant differences in the variances with the Levene's test for the UFH assay ($p = 0.007$) and the LMWH assay ($p = 0.009$), so one-way ANOVA could not be applied. When equal variances were not assumed, the Tamhane test returned significant differences, however UFH at a concentration of 0.25 U/mL resulted in significant error as a result of one outlier causing

insignificant differences between 0.25 U/mL and all other concentrations of UFH. The UFH assay had a statistically significant

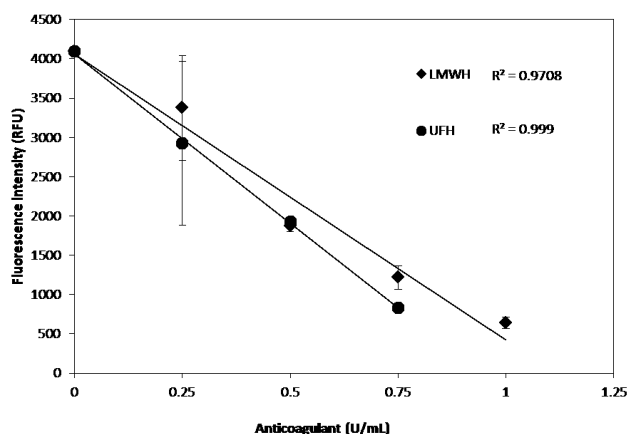


Fig. 5. Dose response curve of human plasma spiked with UFH from 0 – 1 U/mL ($R^2 = 0.97$) and LMWH from 0-1 U/mL ($R^2 = 0.99$) in the anti-Xa device ($n=3$).

range from 0 – 0.75 U/mL. The same pattern was also observed with the LMWH assay where 0.25 U/mL did not appear significantly different from 0 and 0.5 U/mL LMWH and 0.5 U/mL was not significantly different from 0.75 U/mL. This again could be attributed to the significant outlier encountered at 0.25 U/mL which resulted in a CV of 19%. Variability of 12% was also observed at 0.75 U/mL. The LMWH assay had a statistically significant range from 0 – 1 U/mL.

Patient Analysis

To evaluate the integrated device, 10 plasma samples from patients on UFH therapy were tested in the device. For these patients on UFH therapy, the fluorescence responses from all patients were converted into U/mL and correlated with the corresponding aPTT value from the hospital laboratory. The correlation can be seen in Fig. 6.

Fig. 6: UFH correlation: Anti-Xa device and hospital aPTT assay.

The fluorescence intensity value at 30 seconds was calculated for all UFH patient samples. From the data presented in Fig. 6 it can be seen that UFH generates a good correlation with a Pearson coefficient of 0.88. The trend observed is that with increasing fluorescence, the aPTT value decreases which is reflective of decreasing heparin concentrations.

Quite a share of the POC market is occupied by coagulation monitoring devices. Many of these POC diagnostics are based on traditional methods and testing principles, such as clotting time determined by PT, aPTT or ACT (activated clotting time). The adaptation of many of these assays to POC devices was instigated by the significant need for more rapid analysis of coagulation status in emergency room settings.¹⁶ While these devices are commonly used and there is a demand for them, there is certainly scope for the development of novel devices that could improve upon conventional testing methods.

The first move away from standard clot-based testing began in the 1960s with the development and introduction of peptide substrates specific for coagulation proteins such as thrombin and plasmin.^{17,18} Factor Xa was then identified as a significant protein in the coagulation cascade, occupying a pivotal position that could be targeted when monitoring anticoagulant therapy.¹⁹ The development of a FXa specific peptide substrate resulted in the emergence of the anti-Xa colorimetric assay in 1976 which was used to monitor heparin therapy.⁶ Central diagnostic laboratories refer to the anti-Xa assay as the 'gold standard' assay for measuring heparin anticoagulant therapy. Despite its popularity, it has not yet been translated into a POC device.

The work presented here focuses on the development of a POC device for monitoring anticoagulant therapy in cardiac patients that operates on the anti-Xa assay principle. Our initial aim was to develop a simple disposable chip that incorporates the anti-Xa assay. The simplicity of the chip is achieved through the mechanism of capillary fill, so there is no integration of complex, sophisticated, and costly instrumentation such as pumps or valves into the device. The chip was fabricated from 188 μm Zeonor[®] which is easily manipulated during manufacture on the Graphtec Vinyl Cutter. Other microfluidic techniques such as laser ablation or hot embossing can be costly to operate and often require skilled operators, compared to cutting plotters which are user friendly and low cost alternatives.

As aforementioned, COP was chosen as the substrate material for this chip design as it offers additional advantages over polymers such as polycarbonate (PC) and polyethylene terephthalate (PET) in terms of its excellent optical clarity, high UV transmission, easy modification of its surface and suitability for disposable POC devices.²⁰⁻²²

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.²³ Native Zeonor[®] is highly hydrophobic which is suitable for reagent deposition but it becomes problematic when working with devices using capillary fill as it prevents flow. To render the Zeonor[®] surface hydrophilic, three surface chemistries were analyzed to modify the polymer. From the results obtained, the Hydromer-modified Zeonor[®] with low water contact angles of $17.5^\circ \pm 0.9$, produced the most reproducible capillary fill times of

20.3 s \pm 2.1. The device was based on a joint hydrophilic/hydrophobic design to incorporate both the reagent deposition and capillary fill components of the device. The method of detection for this device is based on fluorescence, so Zeonor[®] was selected, as it resulted in low background fluorescence at wavelengths of 342 nm. Treatment with the HY10 coating resulted in responses of 640.9 \pm 2.3 AU, which were too high in terms of background fluorescence.

The fully integrated device developed showed sensitivity in the therapeutic ranges for UFH (0 – 0.75 U/mL) and LMWH (0 – 1 U/mL). Average CVs were <11%, despite high CVs at 0.25U/mL which would be reduced with further device development.

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. Comparisons can be drawn with the Hemochron[®] systems which are some of the most commonly used POC coagulation devices on the market. Hemochron[®] aPTT measurements report linearity with heparin up to 1.5 U/mL, at intervals of 0.25 U/mL and CVs of <14%. Our system is also outperforming other POC diagnostics in terms of volume and turnaround times. The anti-Xa assay and device requires a small sample volume of 20 μ l and results processed within 30 or 60 seconds. This correlates with devices such as the CoaguCheck (Roche Diagnostics, UK), yet significantly lower than the 50 μ l sample volume required for the Hemochron[®] systems.

Conclusion

In terms of patient analysis, plasma samples from patients on UFH were tested on the device and a Pearson correlation of 0.88 was achieved by comparing fluorescence with clotting time. With low heparin concentrations, fluorescence intensities are high and clotting times are short. Here we present preliminary results of a POC anti-Xa device but 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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