DEVELOPMENT OF A BIODIAGNOSTIC DEVICE ASSAY FOR COAGULATION MONITORING

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Abstract: There is an urgent need for the development of reliable point-of-care devices capable of anticoagulant dose monitoring due to the increasing number of patients being treated with clotting control therapy. Millions of patients suffering from cardiovascular-related disorders rely on the anticoagulant therapy. One of the commonly administered drugs is warfarin. It is effective for primary and secondary prevention of venous thromboembolism, for prevention of cardioembolic events in patients with atrial fibrillation or prosthetic heart valves, for prevention of stroke, recurrent infarction or mortality in patients with acute myocardial infarction and for the primary prevention of acute myocardial infarction in high-risk men. Regular monitoring of warfarin effect is of paramount importance and therefore affords are made to develop novel, reliable point-of-care devices for drug level determination. Miniaturized microfluidic systems made of polymers have gained great interest of the diagnostics industry in recent years. Due to the low cost of manufacturing and processing, they have been employed in the development of several disposable diagnostic systems. Among the wide selection of different synthetic polymers, thermoplastics have gained significant popularity. Cyclic polyolefins (COPs) are a relatively new class of thermoplastics with an excellent combination of optical and electronic properties and are dimensionally stable while being subject to a range of operational temperatures and pressures. One such COP is marketed by Zeon Corp. under the brand name Zeonor®. This material has been used as the base for the developed assay. The technology developed by Åmic B.V. (Sweden) allowed the formation of an ordered array of micropillars which introduce controlled and highly reproducible capillary filling forces when liquid samples are introduced to the substrate. Capillary forces play an important role in most of these systems. Assays based on the flow of a fluid in a device with some form of immobilized reagents are considered as the most commonly used tool in many detection systems, including diagnostics. Herein, the concept of monitoring blood clotting properties by measuring a sample distance traveled in a lateral flow system was shown. Substances known to be strong coagulation activators were employed in the monitoring system. All necessary components were incorporated into a test strip, so that no pre-treatment steps were required. These were capable of inducing rapid clot formation and thus arrest of sample flow. The device was shown to be a viable tool for measuring the clotting status of samples containing different quantities of an anticoagulant. This idea of a simple assay device could be employed in a point-of-care determination of a drug level.

1 INTRODUCTION

Close to six million people in the world take oral anticoagulants on a permanent basis, including patients with artificial heart valves or those affected by atrial fibrillation or thrombotic disease. There are also patients who have to rely on them for periods of several weeks or several months (www.roche.com, www.argatroban.com). The number of patients under vitamin K antagonist anticoagulant therapy is expected to reach 10 million globally by 2010 (www.roche.com). Coagulation monitoring and drug dosage adjustment are required to maintain the INR (International Normalized Ratio) within the therapeutic range (Ansell et al., 2004). INR is a system that was established by the World Health Organization (WHO) and the International Committee on Thrombosis and Hemostasis for reporting the results of blood coagulation tests. The WHO procedure is standardized – thromboplastin reagents are calibrated against a standard by means of the International Sensitivity Index (ISI). The INR can be calculated as follows (www.who.int/en):

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$$INR = (\text{observed ratio})^{ISI \text{ of thromboplastin}}.$$

Warfarin (Fig. 1) is one of the most commonly prescribed anticoagulants. It belongs to a group of coumarins that exert their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its 2,3-epoxide (vitamin K epoxide) (Hart et al., 2007, Baigent et al., 1998). Warfarin therapeutic dosage can be affected by several factors and therefore, it can be difficult to manage (Sick et al., 2007). The most common complication of warfarin therapy is bleeding, which occurs in 6 to 39 % of recipients (Levine et al., 1995). The incidence of complications varies within this range, depending upon the clinical indication and the intensity of anticoagulation. Due to the variability in the anticoagulant response to warfarin, regular monitoring and dosage adjustment are required to maintain the INR within the therapeutic range (Hirsh et al., 2003).

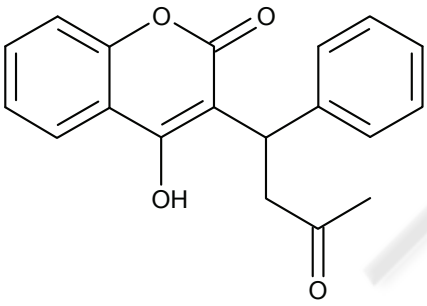

Figure 1: Chemical structure of warfarin.

The development of microfluidic device platforms is already an important area for biomedical device design. Polymer-based microfluidic devices and their associated materials have gained particular interest in recent years. Thermoplastics have gained significant popularity as substrates for the production of disposable devices for biomedical applications having low raw material and manufacturing costs. Their properties which include thermal stability, 'mouldability', precise structural and morphological control over surface properties, chemical and biological inertness, good optical and electrical characteristics and many more are resulting in the replacement of traditional materials such as glass, silicon and nitrocellulose as the foundation of device fabrication.

2 MATERIALS AND METHODS

4Castchips® B2.2 (Fig. 2) were injection molded by Åmic AB (Uppsala, Sweden) in cyclic olefin polymer (COP) (Zeonor 1020R®) to form micropillars (height 65-70 µm, top diameter ca 50 µm, bottom diameter ca 70 µm, the distance between the centres of the pillars in a row 85 µm, the distance between the centres of the pillars in a column 185 µm). These facilitated controlled and highly reproducible capillary filling of liquid samples.

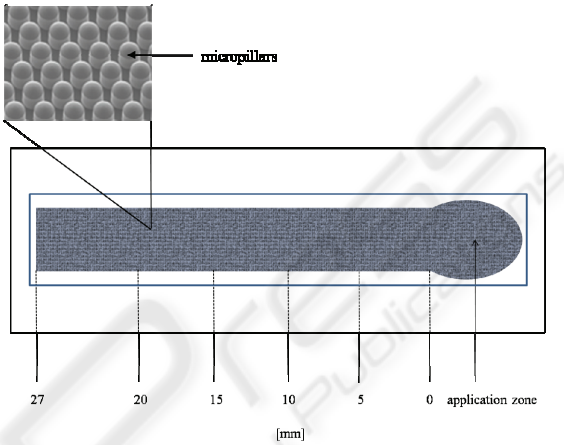

Figure 2: Graphical representation of the B 2.2 micropillar lateral flow device employed for the detection of fibrinogen level. The test channel possessed hot-embossed micropillar structure, as shown in the magnified inset.

The assay platforms were coated with a mixture of activating reagents by a drop-deposition. 10 µL was applied and left to dry in under ambient conditions. Among the active components immobilized on the assay platform were: activated partial thromboplastin time (aPTT) reagent, aPTT-SP (Hemosil), prothrombin time (PT) reagent, Simplastin HTF (BioMerieux) and Russell's Viper Venom (RVV) (Pethapharm). aPTT activator was ready-to-used solution. PT reagent was reconstituted in 2- or 4-fold less volume of diluent than suggested by manufacturer. The activity of RVV solution was 50 U/mL. Positive control was normal clotting, control plasma (Hemosil), while negative control consisted of control plasma supplemented with heparin at a final concentration of 50 U/mL. Such a high concentration of an anticoagulant was used to ensure no clotting occurred. The time required for a test solution to reach each step of a test channel (Fig. 2) was measured. The filling characteristics were assessed on a basis of obtained filling profiles. In addition, the device was validated using anonymous warfarin-treated patient plasma samples with INR values of 1.1, 2.1 and 9.0.

3 RESULTS AND DISCUSSION

3.1 Assay Chemistry Formulation

The development of the assay was based on the monitoring of distance traveled by normal clotting and non-clotting (heparinized) samples. The aim was to achieve a significant difference in a distance traveled between these two variants that would allow identification of minor clotting disorders (slightly prolonged CT). Substances known to facilitate rapid clot formation (aPTT, PT, RVV) were employed in platform development in order to achieve a flow cessation. The distances traveled by normal and heparinized samples on chips coated with a variety of activator combinations are illustrated in Fig. 3.

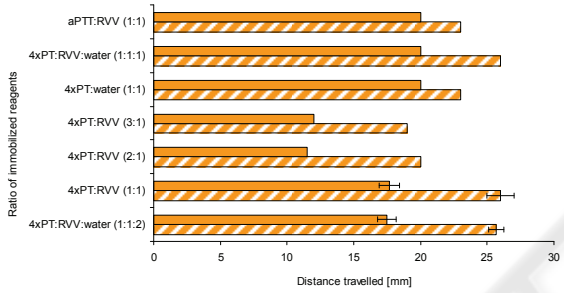

Figure 3: Distance traveled by normal clotting (plain) and non-clotting, strongly heparinized (striped) plasma samples. Test channels were coated with clotting activators at different ratios.

The difference in the distances traveled by clotting and non-clotting samples was between 3 and 8.5 mm depending on a formulation used. The use of the aPTT reagent did not allow rapid clot formation. The difference of 3 mm would not allow a precise differentiation between samples of varying anticoagulant activity. Similarly, 1:1 mixtures of 4-fold concentrated PT with water with or without RVV did not result in good discrimination between samples of different clotting abilities. Dried mixture of 4-fold concentrated PT and RVV at ratios of 3:1 and 2:1 yielded a short distances traveled of 12 and 11.5 mm for clotting and 19 and 20 mm for non-clotting samples. The significant decrease in a distance traveled was probably not an effect of an enhancement in clotting, but was more likely due to high concentration of immobilized PT reagent. The deposition of high protein concentration (tissue thromboplastin) and phospholipids could result in a change of surface properties such as roughness and wettability. It has been noticed that the distance was short not only for clotting sample but also for the negative control sample, for which no clotting occurred. Therefore, the reduction in the distance traveled was of no benefit because of the changes introduced to the surface properties. Formulations composed of 4-fold concentrated PT and RVV with or without water dilution at a ratio of 1:1 or 1:1:2 proved to be best at yielding a significant difference in distance. Fill times were measured at each stage of the channel coated with these formulations. In both situations they looked similar as illustrated in an example in Fig. 4. It has been shown that the filling profile was very similar for clotting and non-clotting samples. The flow of a clotting sample was rapidly arrested at between 15 – 20 mm.

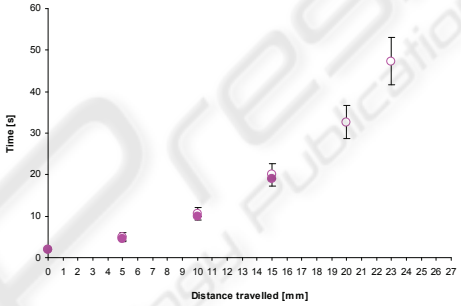

Figure 4: Fill time profiles obtained for normal clotting (filled symbols) and non-clotting (empty symbols) plasma samples tested in a channel coated with 4-fold concentrated PT reagent, RVV and water mixed at a ratio of 1:1:2 (n=3).

3.2 Validation with Patient Samples

The platforms coated with 4-fold concentrated PT + RVV (1:1) and 4-fold concentrated PT + RVV + water (1:1:2) were selected for further validation using patient samples. Three patient samples with different INR values were tested: 1.1, 2.1 and 9.0. Results obtained for normal clotting and heparinized (50 U/mL) plasma samples and for patient plasma samples are shown in Fig. 5. The 1:1 PT:RVV showed good discrimination of INR at the lower range (1.0 to 2.1) but due to assay variability it was unable to discriminate higher values (INR 9.0 and non-clotting controls). However, PT:RVV at 1:1.2 showed poor differentiation between INR 1.0 and 1.1, but was better at distinguishing INR 1.1, 2.1, 9.0 and non-clotting controls. However, variability again made it difficult to distinguish INR 9.0 from other values due to the long and variable times which result.

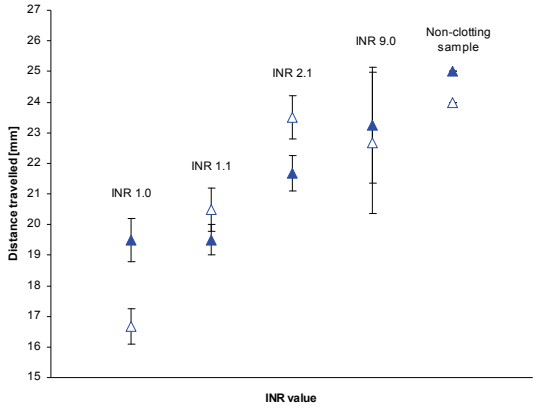

Figure 5: Distances travelled by samples with INR of 1.0 (normal clotting control), 1.1, 2.1, 9.0 and non-clotting. Chips containing 4-fold concentrated PT:RVV at a 1:1 ratio (empty symbols) and 4-fold concentrated PT : RVV : water at 1:1:2 ratio (filled symbols) were used for testing (n=3).

4 CONCLUSIONS

The principle of a point-of-care lateral flow device for the anticoagulant therapy monitoring has been shown. The device platform made of cyclic poly olefin polymer and coated with an optimized mixture of activating agents has been shown a reliable tool for an assessment of blood clotting properties. A mixture of Russell's Viper Venom and Prothrombin Time reagents allowed rapid clot formation which resulted in the cessation of sample flow. Significant differences in the distance traveled between a normal clotting and a strongly heparinized plasma sample were shown. The presence of an anticoagulant (warfarin) in a patient plasma sample delayed clot formation and therefore resulted in a prolonged distance traveled in comparison to a normal clotting control. The device requires further optimization in order to obtained better recognition between samples of different clotting statuses. However, the idea of an anticoagulant dose monitoring using the lateral flow device for the distance traveled measurement was shown to be viable.

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