PRELIMINARY STUDY OF SIMULTANEOUS MULTI-ANTICOAGULANT DEFICIENCY DIAGNOSIS BY FIBER OPTIC MULTI-ANALYTE BIOSENSOR

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Abstract: Protein C (PC), protein S (PS), antithrombin III, and plasminogen are four important anticoagulants in blood plasma. Deficiency of any of these biomolecules may lead to thrombo-embolic complications including lung embolism, heart attack, and stroke. A multi-factor sensing system is beneficial for identifying the cause of abnormal blood clotting more effectively, rapidly, and cost-effectively. As an initial effort toward simultaneous multi-anticoagulant detection, a PC and PS dual-sensing system has been under development in our research group.

A fiberoptic PC biosensor utilizing fluorophore-mediated sandwich immunoassay was already developed for rapid (~5 minutes) PC deficiency diagnosis. After a single PS sensor was developed for the PS deficiency diagnosis, the two sensors were connected in series to form a dual-sensing system. The cross-reactivity between the analytes and the sensors was found to be minimal. For easier sensing operation, a mixture of fluorophore-linked anti-PC and anti-PS was applied. The results showed that the mixture can be used with a slight signal reduction. When PC and PS was mixed in a sample, the signal intensity was decreased by approximately 5% for both sensors. A study is currently being performed to overcome the signal reduction by increasing the flow velocity and incubation time.

1. INTRODUCTION

Hemostasis maintains blood fluidity within blood vessels (anticoagulation) and, at the same time, prevents blood loss by immediately sealing defects (coagulation) on the injured vessel wall.[1] When the anticoagulation capacity is impaired, venous thrombo-embolism (VTE) complications including thrombosis, lung embolism or heart attack may occur due to the hindrance in oxygen and nutrient transport to tissues.[2]

Table 1. Frequency of hemostatic defects leading to VTE.

Hemostatic Defect	Freq. in General Population	Freq. in VTE Patients
APC resistance*	3%-7%	20%-40%
AT deficiency	0.02%	5%-6%
PC deficiency	0.2%-0.4%	5%-6%
PS deficiency	0.1%	5%-6%
PLG deficiency	-	1%

* A single point mutation of the coagulant Factor V.[6]

The annual incidence of VTE is approximately 1 in 1000 persons, and 50,000-100,000 deaths occur each year in the US.[3] Table 1 lists the inherited hemostatic defects that cause VTE.[4] Among them, the most frequent (20%-40%) is the resistance to activated protein C (APC resistance). It is mainly caused by a single point gene mutation of human Factor V, a coagulation factor: the mutated factor does not become inactivated by the activated protein C (APC).[5, 6] The other defects, deficiencies of PC, protein S (PS), antithrombin III (AT), and plasminogen (PLG), share a similar nature: insufficient amount of anticoagulant proteins in plasma. Altogether, these defects account for about 15%-20% of patients with VTE. Compared with the subjects with APC resistance, their risk of thrombosis is higher and their frequency of deep vein thrombosis is more (88% in PC, 90% in AT, 100% in PS deficiency, and 57% in APC resistance).[7] Therefore, accurate diagnostic tools for these anticoagulant deficiencies are invaluable. In addition, a multi-factor sensing system is very beneficial not only to identify the actual cause more effectively and quickly, but also to reduce costs for diagnoses.

Due to the low level of anticoagulants in plasma (~nM), sensing methods for these factors need to be highly attuned. In addition, the presence of many homologues to these proteins in plasma requires a highly specific assay. Currently, the diagnosis for these factor deficiencies is performed by enzyme linked immunosorbent assay (ELISA). Although very accurate, ELISA is time-consuming (hours to a day), expensive, and technically complicated. A fiber optic biosensor, performing a sandwich, fluorophore-mediated immunoassay on the fiber surface, can provide an accurate, rapid, cost-effective, and user-friendly deficiency diagnostic method. The detector for this sensing system is a compact fluorometer, capable of monitoring four optical fiber sensors simultaneously.

As an initial effort toward the development of a simultaneous multi-anticoagulant detection system, the feasibility of a PC and PS dual-sensing system was explored using a two-channel fiber optic biosensing system. A fiber optic PC biosensor has already been developed for rapid (~5 minutes) PC deficiency diagnosis.[8-15] PS is a co-factor for PC activation and, therefore, an important anticoagulant. The heterozygous, PS-deficient patients have 15%-50% of the normal level of functional PS (10 μg/ml), which is 1.5-5 μg/ml (20-70 nM).[1]

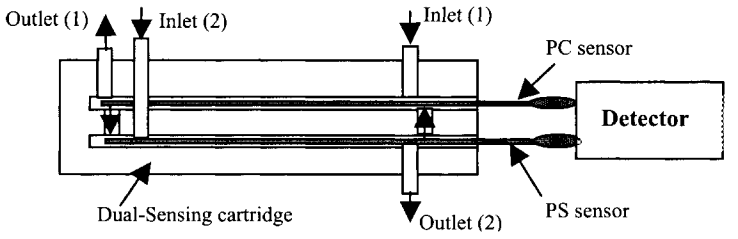

Figure 1. Schematic diagram of the dual-sensing unit.

2. MATERIALS, INSTRUMENTS, AND METHODS

2.1. Materials and Instruments

Human PC (MW = 62,000) and two different kinds of mouse monoclonal anti-human PC were provided by the American Red Cross (Rockville, MD). Human PS (MW = 70,000) and different types of mouse monoclonal anti-human PS were purchased from Haematologic Tech (Essex Junction, VT). Cy5 reactive dye was from Amersham Pharmacia Biotech (Uppsala, Sweden). Fluorometer, Analyte 2000^{TM}, and quartz optical fibers were obtained from Research International (Monroe, WA).

2.2. Methods

Optical fibers 6 cm in length were chemically treated, and, primary monoclonal antibodies (1° Mabs) against PC and PS were immobilized on the surface of PC and PS fibers, respectively, via an avidin-biotin bridge.[11] Then, the fiber was inserted into a glass chamber (100 ml) and hot glued to form a functional sensor unit.[11] For the dual-sensing unit, a PC and a PS sensor were placed in series (Fig. 1). Conjugation of Cy5 with secondary monoclonal antibodies (2° Mabs) was performed according to manufacturer's instructions. PC and PS samples were prepared in the emulated human plasma (103 mg-HSA/ml- phosphate buffered saline).[13] During the assay, samples (1 ml) and Cy5-2° Mab were circulated through the sensing chamber at a pre-determined flow velocity for 0.5 and 2 min, respectively.[14]

3. RESULTS AND DISCUSSION

Each data point in figures is presented with the mean value and standard deviation of multiple measurements (> 3 times) on at least two sensors, unless otherwise stated.

3.1. PS Biosensor Development

The molar concentration of the PS sensing range (20-70 nM) is greater than that of the PC (8-40 nM). Therefore, the incubation times for the PC sample and Cy5-2° Mab (0.5/2 min) were expected to be sufficient for the PS sensing. The effect of the flow

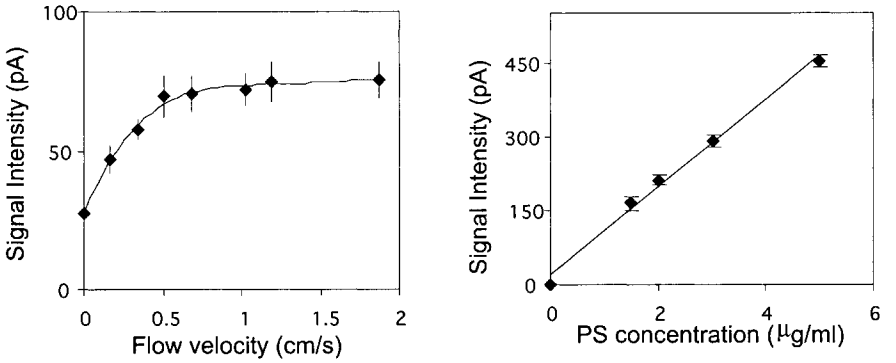

Figure 2. **A**: Effect of flow velocity on PS sensor performance (1.5 μg/ml PS). **B**: Sensitivity of the PS sensor at a flow velocity of 0.5 cm/s.

velocity on the PS sensing performance was investigated to determine the minimal flow velocity.

PS samples at 1.5 mg/ml (the lower sensing limit) and Cy5-2° Mab-PS were applied (i.e., circulated) at various flow velocities between 0.1 and 2 cm/s in a sensor unit. As Figure 2A shows, the signal intensities increased linearly up to the flow velocity of 0.5 cm/s (increase by 153%, compared with the static conditions). As the velocity became higher than 0.5 cm/s, the signal increase slowly tapered, showing the changes in the reaction kinetics from the mass-transfer-limited to the reaction-limited. The minimum effective velocity for the PS sensing was determined to be 0.5 cm/s. This was less than the optimal velocity of the PC sensing (0.7 cm/s for 1 mg/ml PC),[15] possibly because of the higher PS sensing range.

Using this minimum effective velocity, the sensitivity of the PS sensor in the sensing target range was tested (Fig. 2B). The signal intensity increased linearly, with the PS concentration over the range tested with a correlation cofficient of 0.99.

3.2. Effect of the Mixture of Two Second Antibodies on Sensing Performance

For dual factor sensing after the sample incubation, Cy5-linked antibody against PC (Cy5-2° Mab-PC) and Cy5-linked antibody against PS (Cy5-2° Mab-PS) need to be introduced into the respective sensor chambers. This can be done by either applying each antibody to the respective chamber, as shown in Figure 1 (inlet 1 and outlet 1 for PC sensor; inlet 2 and outlet 2 for PS sensor), or by running a mixture of two different Cy5-2° Mabs through both sensors [Fig. 1 (inlet 1 and outlet 2)], if Cy5-2° Mabs do not cross-react. The second case was tested first because it requires an easier design for the fluid direction manipulation. The mixture was applied to each sensor to investigate the extent of cross-reactivity.

Figure 3A shows the signal intensities generated by a PC sensor using only Cy5-2° Mab-PC (■) and the Cy5-2° Mab mixture (◊). Both measurements were performed by a single PC sensor to eliminate possible fiber-to-fiber sensing variation. The signal intensities generated by the mixture slightly decreased, but the reduction was within 6%, with a similar standard deviation for both (5%-10%). A possible cause for the signal

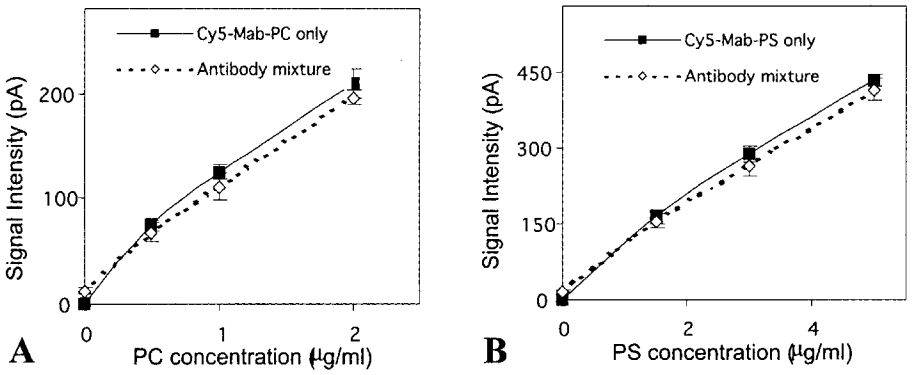

Figure 3. **A**: Effect of the Cy5-2° Mab mixture on the PC sensor performance. **B**: Effect of the Cy5-2° Mab mixture on the PS sensor performance.

reduction could be that the presence of another type of molecule causes a slower mass transfer rate. The background signal of the Cy5-2° Mab mixture slightly increased (10-15 pA) for all tested concentrations, compared with that of Cy5-2° Mab-PC only (not shown).

The effect of the antibody mixture on PS sensor performance was also studied (Fig. 3B), and the signal intensities with the mixture were decreased slightly, with a maximum of 4.5%. The background signal with the mixture was also increased by 10-15 pA.

These results demonstrated that the quantification of PC and PS with the mixed Cy5-2° Mabs can be as accurate as that of the respective Cy5-2° Mab, if the standard curve is also obtained with the mixed Cy5-2° Mabs. The actual cause of the signal reduction is currently being investigated.

3.3. Preliminary Study of Simultaneous PC and PS Detection

A PC sensor and a PS sensor were placed in the dual-sensing unit for simultaneous detection. Samples with the mixture of PC and PS were applied through the two sensor chambers at a flow velocity of 0.7 cm/s (the optimum for the PC sensor). Figure 4A shows the signal intensities resulting from 10 µg-PS/ml-plasma on the PC sensor and 4 µg-PC/ml-plasma on the PS sensor, respectively, in the dual-sensing unit. The response from the PC sensor is less than 1% of that from the PS sensor, indicating a minimal PS cross-reactivity with the PC sensor at the maximum PS concentration. The PS sensor also demonstrated a minimal cross-reactivity with the 4 µg/ml PC.

To investigate the interference of PS molecules to the PC sensor during simultaneous, dual factor detection, two samples were prepared: a sample of 0.5 µg-PC/ml-plasma (the lower sensing limit for PC); in another sample, PS was added at 10 µg/ml (the normal PS concentration in plasma) to 0.5 µg-PC/ml-plasma. Again, the measurements were performed on the same sensor (Fig. 4B). The signal intensity from the factor mixture was decreased by 10%, compared with the intensity of the sample with

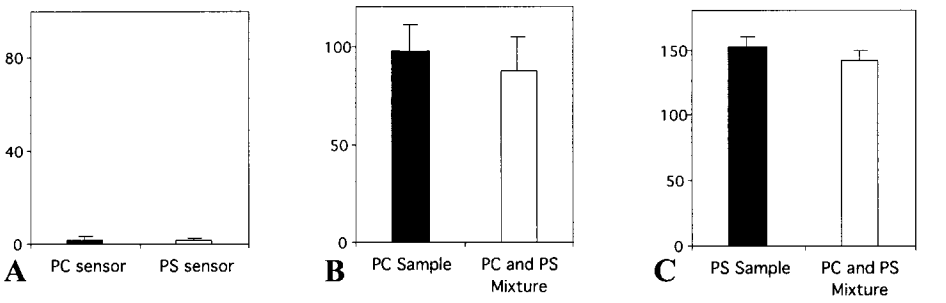

Figure 4. A: Cross-reactivity in the dual-sensing unit [10 μg/ml PS over the PC sensor and 4 μg/ml PC over the PS sensor; Cy5-2° Mab mixed]. **B**: Effect of PC and PS mixture on the PC sensing [dual-sensing unit; (0.5 μg/ml PC) and (mixture of 0.5 μg/ml PC and 10 μg/ml PS); Cy5-2° Mab mixed]. **C**: Effect of PC and PS mixture on the PS sensing [dual-sensing unit; (1.5 μg/ml PS) and (mixture of 1.5 μg/ml PS and 4 μg/ml PC); Cy5-2° Mab mixed].

PC only. Similarly, a mixture of 1.5 μg-PS/ml-plasma (the lower sensing limit for PS) and 4 μg-PC/ml-plasma (the normal concentration) was tested to investigate the interference of PC molecules to the PS sensor (Fig. 4C). The signal intensity generated by the PC and PS mixture was decreased by 7%. Compared with the PC sensor, PC interference to the PS sensor appeared to be less. We speculate that the reason for this lower amount of reduction in signal intensity is that the greater PS concentration (as high as 10 μg/ml) caused more hindrance in the mass transfer for the much lower PC concentration (0.5 μg/ml).

Unlike the Cy5-2° Mabs interference, the PC molecule interference to the PS sensing (and *vice versa*) will have to be studied more carefully because some patients may have deficiencies of more than one factor.

4. CONCLUSIONS

A fiber optic PS biosensor was developed to quantify functional PS in plasma in the deficiency range, and the feasibility of fiber optic two-channel biosensing system was demonstrated for PC and PS dual-sensing. The mixture of Cy5-2° Mab-PC and Cy5-2° Mab-PS has shown a minimal cross-interference to the PC or PS sensor. Therefore, the mixture can be applied through the dual-sensing unit for PC and PS detection with an easier operation. The cross-reactivity between PC and PS sensors in the dual-sensing unit was demonstrated to be minimal (less than 1%), with 4 μg/ml PC and 10 μg/ml PS (the normal concentrations). Samples with mixed PC and PS were applied to the dual-sensing unit to test the interference caused by the other analyte. When 10 μg/ml PS was added to 0.5 μg/ml PC, a signal reduction of 10% was shown for the PC sensor. When 1.5 μg/ml PS was mixed with 4 μg/ml PC, the reduction was 7% for the PS sensor. A faster flow velocity and/or a longer sample incubation time may overcome the interference.

Other future work includes the integration of micro-electro-mechanical systems (MEMS) technology for the development of a smaller, more cost-effective, automatic, and multi-anticoagulant sensing cartridge. For the future clinical practice, since the sample volume requirement for our sensing system is less than 1 ml, micro-filtration

syringes will be explored to first separate plasma from cellular components in whole blood samples of patients, and then to inject the separated plasma into the sensing unit.

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