Fluorophore-mediated, fiber-optic, multi-analyte, immunosensing system for rapid diagnosis and prognosis of cardiovascular diseases

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Abstract. A prototype of a fiber-optic, multi-analyte, immunobiosensing system was developed to simultaneously quantify disease-representing biomarkers in blood plasma. This system was for simultaneous quantification of two different groups of multi-biomarkers related to cardiovascular diseases (CVD): anticoagulants (protein C, protein S, antithrombin III, and plasminogen) for deficiency diagnosis; and cardiac markers (B-type natriuretic peptide, cardiac troponin I, myoglobin, and C-reactive protein) for coronary heart disease diagnosis. As an initial effort towards the development of a disposable and easy-to-use sensing cartridge as a rapid diagnostic tool for CVD related diseases, a prototype of a flow control system was also developed to automatically perform simultaneous four-analyte quantification. Currently, the system is capable of quantifying the multiple anticoagulants in their clinically significant sensing ranges within 5 minutes, at an average signal-to-noise (S/N) ratio of 25. A simultaneous assay of the four cardiac markers can be performed within 10 min, at an average S/N ratio of 20. When this highly portable multi-analyte sensing system is completed and successfully tested for CVD patient's plasma, it can provide rapid (<10 min) and reliable diagnostic and prognostic information at a patient's bedside. [DOI: 10.1117/1.2192529]

Keywords: cardiovascular disease; coronary heart disease diagnosis; anticoagulant deficiency; cardiac markers; immunoassay; fiber-optic multi-sensing.

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1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in the US with an average of one death every 34 sec and approximately $400 billion of direct and indirect cost in the US alone.[1] Among the CVD related diseases, coronary heart disease (CHD) is the single largest killer of Americans. Early and accurate diagnosis of CHD, especially in the emergency room, is crucial to design an appropriate patient care strategy. By measuring the levels of particular biomolecules, i.e., cardiac markers, in the blood, emergency room physicians can quickly determine whether patients have actually suffered a coronary event. Several important cardiac marker proteins have been identified and routinely used in the current clinical practice. Cardiac troponin I (cTnI) is widely used as a standard biomarker based on its absolute cardiac specificity and its long serum half-life (7–10 days; ESC/ACC, 2002). A fast increase of myoglobin (MG) level in bloodstream following heart attack allows for a rapid patient evaluation. B-type natriuretic peptide (BNP) is useful for the emergency diagnosis of heart failure and for the prognosis in patients with acute coronary syndromes (ACS).[2,3] C-reactive protein (CRP) is an important prognostic indicator of CHD and ACS.[3] A simultaneous quantification of these four cardiac markers allows clinicians to diagnose CHD quickly and to accurately design a patient care strategy, especially in an emergency room.[3] A fast and reliable detection of these cardiac markers will also help medical professionals differentiate diseases among those showing similar symptoms. Maisel et al. have evaluated the value of rapid bedside measurement of plasma BNP to distinguish coronary heart failure from a pulmonary cause of dyspnea for patients presenting a major complaint of acute dyspnea in the emergency room.[2]

The third leading cause of CVD in the US is venous thrombo-embolism (VTE) including deep vein thrombosis, lung embolism, cerebral venous thrombosis, and purpura fulminans. The incidence of VTE is approximately 1 per 1000 persons (1% for the elderly), and it accounts for 100,000 to 150,000 deaths annually.[4,5] When the haemostatic system in human body is unregulated, due to either coagulation problem or more commonly, impaired capacity of natural anticoagulant mechanism caused by anticoagulant deficiencies, the body has a predisposition to fatal venous thrombo-embolism.[6–8] Protein C (PC), protein S (PS), antithrombin III (ATIII), and plasmi-

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Table 1 Clinically significant sensing ranges of the anticoagulants and cardiac markers.

	Biomarkers	Normal Concentration [μg/ml (nM)]	Target Sensing Range [μg/ml (nM)]
Anticoagulant	PC	4 (64)	0.5–2.5 (8–40)
	PS	10 (143)	1.5–5 (21–70)
	ATIII	150 (2300)	45–105 (700–1600)
	PLG	200 (2000)	60–120 (600–1200)
Cardiac marker	BNP	<0.0001 (0.026)	0.0001–0.001 (0.026–0.26)
	cTnI	<0.001 (0.04)	0.0007–0.007 (0.03–0.3)
	MG	<0.03 (1.7)	0.07–0.7 (4–40)
	CRP	<0.8 (6.4)	0.7–7 (5.6–56)

nogen (PLG) are four major anticoagulants in blood.[9] Deficiency in PC, PS, or ATIII was reported to significantly increase the risks of VTE.[10] Altogether, they account for about 15–20% of VTE cases.[11] Therefore, accurate diagnostic tools for early detection of these deficiencies are invaluable to prevent the fatal VTE complications. A simultaneous quantification of these four anticoagulants in blood is beneficial for the accurate diagnosis of the actual cause of an abnormal clotting.

Due to the extremely low levels of these biomarkers (pM ~ nM; Table 1) and the presence of other structurally similar biomolecules in blood, a frequently used assay method is enzyme linked immunosorbent assay (ELISA). Although very accurate, it is time-consuming (hours to days), expensive, and technically complicated. Commercially available test kits for anticoagulants (Sigma; St. Louis, MO), BNP (Biosite; San Diego, CA), cTnI (Roche; Basel, Switzerland), and CRP (Dade Behring; Deerfield, IL) can provide fast, easy, and point-of-care assays. However, they usually provide only qualitative single biomarker information and most of the assay kits are rather expensive. The need for an accurate, rapid, and cost-effective disease diagnosis and prognosis has been the motivation for developing a fluorophore-mediated, fiber-optic immuno-biosensing system. This technique performs a fluorophore mediated sandwich immunoassay within the evanescent field on the surface of an optical fiber,[12] and has been successful for rapid clinical diagnostics.[13–15] In our research group, a fiber-optic immuno-biosensing system was developed for rapid (~5 min) PC deficiency diagnosis.[16–23] Compared to the conventional analytical methods such as DNA analysis, polymerase chain reaction, and ELISA, the immuno-optical biosensor has the advantages of rapid response time, user-friendliness, and cost-effectiveness.[24]

For simultaneous quantification of multi-cardiac-markers or multi-anticoagulants, a single fiber-optic biosensing system needs to be expanded to multi-analyte detection. A common method for multi-analyte biosensing is using multiple labels, such as multiple radioactive markers,[25] fluorophores,[26] or enzymes.[27] However, a loss of sensitivity in the multi-analyte format is usually observed, compared to the individual assay format, due to the poor discrimination of signals generated by distinct labels. Another approach is by spatially separating the assay zone with same or different labels. The simultaneous quantification of multiple analytes in one sample is usually carried out on an antibody array format where different antibodies specific for each of the targeted analytes are immobilized in discrete areas.[28] A simple approach for multi-analyte detection with spatially resolved methods may be connecting the single analyte assay system in series. Bakaltcheva et al. have reported multi-explosive detection using fiber optic biosensing.[29] RAPTOR™, manufactured by the Research International, Inc. (Monroe, WA), uses four polystyrene fiber sensors and is capable of simultaneous monitoring of four analytes.[30] For our application, four individual sensors bearing antibodies specific for each biomarker are placed, in series, in an analyte sensing unit, similar to RAPTOR™. We, however, use quartz fibers and immobilize the first antibodies via biotin-streptavidin reaction to achieve low background signals and high sensor reusability.[16–18] The convective flow application during the incubation of the sample and other reagents was also employed in our system to enhance the mass transport of these chemicals from the bulk media to the sensor surface. Hence, a special microfluidic unit was added for flow velocity control. In addition, a novel fluorescence enhancer developed in our research group is currently used in our sensing protocol that is not compatible with plastic fibers. Our multi-analyte sensing system consists of a sensing unit with four sensors, a fluorometer, and an automatic flow control unit. The results on using this system for the simultaneous quantification of multi-anticoagulants and multi-cardiac-markers with the application of the fluorescence enhancers are presented in this paper. The optimization of the sensing protocol was also performed to realize a rapid and accurate sensing system. To make the sensing procedures more user-friendly and more consistent with minimal human errors, automation of this multi-sensing system operation was also attempted.

2 Materials and Methods

2.1 *Materials*

Human B-type natriuretic peptide (BNP; a peptide of 32 amino acids) was purchased from Bachem (Torrance, CA) and

two different murine, monoclonal IgG against human BNP, from Strategic Biosolutions (Newark, DE). Purified cardiac troponin I (cTnI; MW=29,000), myoglobin (MG; MW =16,000), and C-reactive protein (CRP; MW=114,000) from human heart and the respective, two different murine, monoclonal antibodies were obtained from Fitzgerald Industries (Concord, MA).

Human protein C (PC; MW=62,000) and two types of murine monoclonal anti-human PC (1°Mab-PC and 2°Mab-PC; MW=150,000) were provided by the American Red Cross (Rockville, MD). PC-free human plasma containing less than 1% PC was obtained from American Diagnostica, Inc. (Greenwich, CT). Human protein S (PS; MW=70,000), antithrombin III (ATIII; MW=65,000), plasminogen (PLG; MW=92,000) and two different types of murine monoclonal antibodies against respective anticoagulants (1°Mabs and 2°Mabs; MW=150,000) were purchased from Haematologic Technology (Essex Junction, VT).

Human serum albumin (HSA; MW=68,000), ImmunoProbe™ Biotinylation kit, and Sigmacote® were from Sigma (St. Louis, MO). Fluorolink™ Cyanine 5 (Cy5) reactive dye (the maximum excitation and emission at 649 and 670 nm, respectively) and Alexa Fluor 647 reactive dye (AF647; the maximum excitation and emission at 650 and 668 nm, respectively) were from Amersham Pharmacia Biotech (Uppsala, Sweden) and Molecular Probes (Eugene, OR), respectively. Plastic clad silica (PCS) optical fibers (600-μm core diameter) were purchased from Research International, Inc. (Monroe, WA). LabView™ software package (version 7.1) and data acquisition card (6024E PCMCIA) were obtained from National Instruments (Austin, TX). Electronically controllable micro peristaltic pumps (35 rpm, 12 VDC, 140 mA) and 2-way (normally open) and 3-way micro solenoid valves (12 volts DC) were from APT Instruments (Litchfield, IL) and ASCO Scientific (Florham Park, NJ), respectively.

2.2 *Methods*

2.2.1 *Samples and reagents preparation*

The previous study demonstrated that a 103-mg-HSA/ml-PBS solution can be used to emulate analyte-free human plasma with the same viscosity (1.9 cP).[18] Therefore, the sample was prepared by adding a known amount of the analyte (cardiac markers or anticoagulants) to the HSA solutions at 103 mg/ml. Conjugation of biotin to 1°Mabs was achieved using the ImmunoProbe™ Biotinylation kit and the conjugates were separated from free biotin by the size exclusion chromatography (Amersham-Pharmacia Biotech; Piscataway, NJ) with P-10 Gel (cutoff MW=20,000), according to manufacturer's instructions. 2°Mab was reacted with bisfunctional NHS-ester Cy5 dye for 30 min to obtain the optimum ratio of 1:5 and the Cy5-2°Mab conjugate was separated from unreacted dye by the size-exclusion chromatography.[17] For the Alexa Fluor 647 (AF647), the reaction time was 1 hour, following the manufacturer's instructions. Sensor washing buffer (PBST; pH 7.4) was PBS buffer with 0.01% (v/v) Tween 20. Sensor regeneration buffer was 0.1 M triethylamine solution at pH 11.[21,31]

2.2.2 *Fiber-optic biosensor preparation*

An optical fiber (Research International; Monroe, WA) was tapered to maximize the internal reflection of photons within the fiber.[12] The cladding of an optical fiber was removed at a predetermined length from one distal end. The exposed fiber optic core was tapered in hydrofluoric acid using the automatic tapering machine for 1 h.[31] Next, the tapered fiber surface was chemically treated to be streptavidin-coated, as described by Bhatia et al.[32] in case a capillary was used as a sensing chamber. Before inserting the fiber into the chamber, Sigmacote® was applied to its inner surface to form a tight film of silicone on glass to minimize analyte adsorption. The chamber is connected to two nylon T connectors at both ends to form a functional sensor as described by Spiker et al.[21] Biotin-conjugated 1°Mab (65 μg/ml) was then injected into the sensing chamber and incubated at 4 °C for 24 h. The 1°Mab was immobilized via the avidin-biotin bridge on the fiber surface.[22] 1°Mab for respective analytes was immobilized on the fiber surface to form functional sensors. Before performing assays, 0.1 M ethanolamine was applied as a blocking buffer to minimize possible nonspecific bindings of biomolecules. If not used immediately, the sensor was stored at 4 °C in phosphate buffered saline (PBS; pH 7.4) with 0.02% (w/v) sodium azide.

2.2.3 *Assay procedures*

During an assay, the antibody-coated sensors were connected to the fluorometer, Analyte 2000™ (Research International; Monroe, WA). It has four channels capable of simultaneously quantifying four factors. The assay procedures with static incubation (i.e., no flow during sample/reagent incubation) were performed as described by Spiker and Kang, unless otherwise specified.[20,22] For the assay with convection, during reaction (incubation), sample and reagent solutions were injected into the sensor unit via a peristaltic pump (Ismatec, Inc.; Glattbrugg-Zürich, Switzerland) and circulated within the sample circulation unit at a predetermined flow velocity.[33] Currently, the total sample volume for an assay for four analytes is approximately 1 ml. The assay procedures are briefly described as follows:

- step 1: sample injection and incubation;
- step 2: washing the sensor to remove non adsorbed molecules;
- step 3: AF647-2°Mab injection and incubation;
- step 4: washing the sensor to remove non reacted AF647-2°Mab; and
- step 5: regenerating the sensor using the regeneration buffer, for a next assay cycle.

At the end of steps 2 and 4, readings were taken using the Analyte 2000™, where optical signal is converted to photocurrent (pA). The difference in the signal intensity between these two steps (ΔpA) is a direct measure of the fluorescence produced by the 1°Mab/analyte/AF647-2°Mab complex on the sensor surface, which is correlated with the target analyte concentration in the sample.

For multi-analyte quantification, four sensors were placed in the four-analyte sensing unit (Fig. 1). Each sensor was inserted into one of the four microchannels which were connected in series. The sensing procedures were the same as the individual analyte quantification.

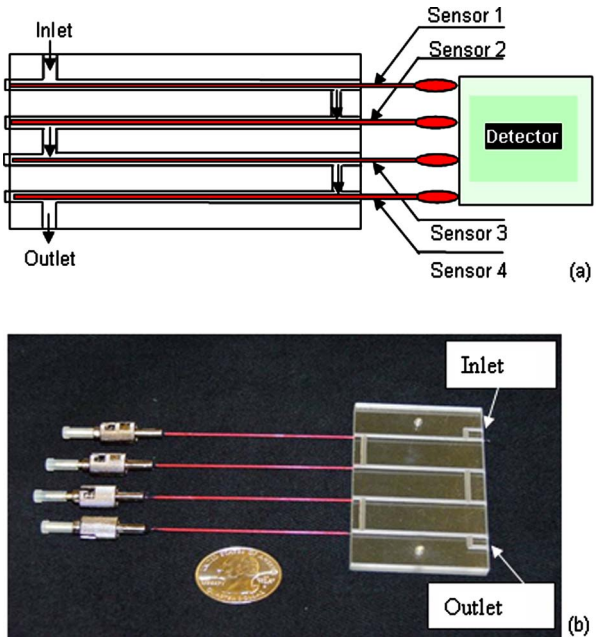

Fig. 1 (a) Schematic diagram and (b) photograph of a prototype four-channel sensing unit with four 3-cm sensors inserted into the channels, each (designed by Kang, et al.; the microchannels are micromilled by Keynton et al., University of Louisville).

3 Results and Discussion

3.1 *Multianticoagulant Sensing System*

As stated in Table 1, the target sensing ranges for the anticoagulants are: PC (0.5∼2.5 μg/ml; 8∼40 nM), PS (1.5∼5 μg/ml; 21∼70 nM), ATIII (45∼105 μg/ml; 700∼1600 nM), and PLG (60∼120 μg/ml; 600∼1200 nM). Individual fiber-optic biosensors were developed separately and optimized. For a multi-analyte sensing, four sensors were connected in the four-analyte sensing unit and simultaneous quantification of four analytes was performed.

3.1.1 *Single sensor study with protocol optimization*

Blood plasma is frequently the sample for an assay in the clinical practice. When the incubation time for the sample and reagent was 5 and 3 min, respectively, the signal intensity generated by PC molecules in plasma sample was decreased by approximately 70%, compared to that in the buffer based sample.[18] Theoretical and experimental analyses demonstrated that the main cause for the signal reduction is the high viscosity of plasma.[17,31] The reaction kinetics of analyte (here, PC) and 1°Mab in a fiber-optic biosensing system involves two steps: the analyte transport from the liquid medium to the sensor surface; and the reaction between the transported analyte and the 1°Mab molecule on the surface. During the static incubation period, analyte molecules are transported to the sensor surface by diffusion. The analyte diffusion coefficient (D;cm^2/s) is inversely related to the sample medium viscosity:[34]

$$D = \frac{kT}{6\pi\mu R_A}, \tag{1}$$

where k is the Boltzmann constant (1.38×10^{-23} J/K); T is the sample medium temperature (room temperature, 298 K); μ is the sample medium viscosity (1.0 cP for PBS buffer and 1.9 cP for plasma); and R_A is the radius of the analyte. Since the viscosity of plasma (1.9 cP) is almost twice that of the PBS, the PC diffusion coefficient in plasma is approximately 50% less. Kwon has used the Damkohler number (N_{Da}) for analyzing reaction kinetics: a system with an N_{Da} value of greater than 100 is considered to be diffusion-limited; less than 0.01, reaction-limited.[31,35,36] The values for the PC systems in both PBS and plasma samples are on the order of 100, indicating that the systems are diffusion-limited and the system with plasma samples is more diffusion-limited than the one with PBS buffer.

To enhance the sensing performance by improving the analyte mass transport to the sensor surface, another mechanism, in addition to diffusion, was sought. By applying convective flow during the sample incubation, the PC sensor performance for viscous samples (e.g., plasma) was significantly enhanced.[17] Kwon has analyzed the effect of the convective flow on the fiber-optic biosensors using the thin film theory.[31,36] On the surface of a sensor, there is a film resistant to the analyte mass transport from the bulk solution to the sensor surface to react with 1°Mab immobilized on the surface. The thickness of this film (δ; cm) is closely related to the analyte mass transport rate:

$$\delta = \frac{D}{k_m}, \tag{2}$$

where k_m is the effective mass transfer coefficient (cm/s). When a convective flow is applied during the incubation (reaction) of sample, the film thickness is significantly reduced. For the PC sensing system, compared to the system with the convection at 0.1 cm/s, the k_m value at 0.7 and 1.2 cm/s was increased by 157 and 232%, respectively. As a result, the film thickness was reduced with the increase in the flow velocity. At 0.1 cm/s, the thickness was 12×10^{-3} cm. When the flow velocity was increased to 0.7 cm/s, the film thickness was reduced by 60% (4.5×10^{-3} cm). At 1.2 cm/s, the thickness was only 30% (3.5×10^{-3} cm) of that of 0.1 cm/s. As shown in the experimental analysis, the signal intensity at 0.7 cm/s increased by 110% for 1-μg-PC/ml-plasma, compared to that with the static incubation.[23,31] At very higher flow velocities, although the PC mass transport is faster, the reaction rate is not fast enough to consume all of the PC molecules transported to the sensor surface. Therefore, the signal intensity remained steady at the velocities higher than 0.7 cm/s. The optimal flow velocity for the PC sensing was then determined to be 0.7 cm/s, where the reaction kinetics changes from the mass-transport-limited to the reaction-limited. At this velocity, the PC assay time was reduced to 5 min including 0.5 and 2 min for the sample and second antibody incubations, respectively.[23]

During the initial development of the anticoagulant sensor, fluorophore Cy5 was used as the signal mediator. However, the photobleaching and low quantum yield of this fluorophore have been issues for improvement. Alexa Fluor 647 (AF647)

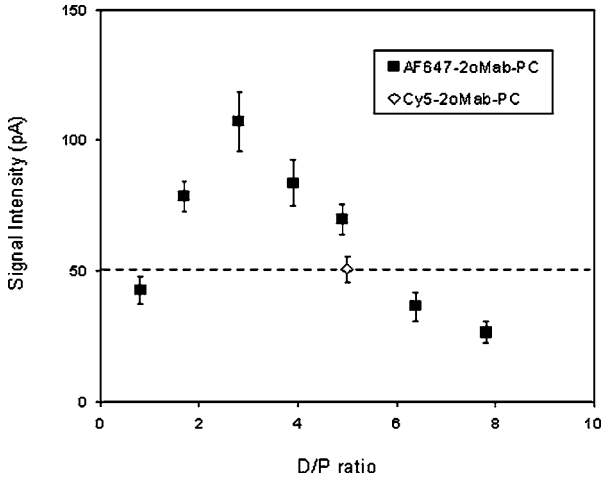

Fig. 2 Fluorescence of AF647-2°Mab-PC in the PC sensing system with change in the D/P ratio and comparison to that of Cy5-2°Mab-PC at D/P ratio of 5 (experimental conditions: 6-cm PC sensors; 1 μg/ml PC in plasma; 5 μg/ml 2°Mab-PC; 0.5/2 min incubation times for the sample and AF647-2°Mab-PC; 0.7 cm/s convective flow velocity).

has a quantum yield more than twice that of Cy5 (0.28) and is more photostable. The excitation and emission wavelengths of AF647 are also compatible with our fluorometer, Analyte 2000™ and it has been demonstrated that AF647 can improve the sensing performance of the fluoro-immunoassays significantly.[37] Therefore, a study was performed to investigate the efficacy of the AF647-conjugated second antibody for the PC sensing. AF647 was conjugated with the 2°Mab-PC (AF647-2°Mab-PC) at various dye-to-protein (D/P) ratios from 1 to 8. 1 μg/ml PC was reacted with the 1°Mab-PC immobilized on the sensor surface and then AF647-2°Mab at various D/P ratios was applied to the sensor (Fig. 2). The signal intensity increased steadily up to the D/P ratio value of 3 and then, at above 3, decreased with the increase in the ratio. Since the fluorescence for free forms of AF647-2°Mab at higher D/P ratios increased (data not shown), one possible reason for the signal intensity reduction in the PC sensing system is the reduced relative affinity of AF647-2°Mab to the PC at higher D/P ratios. The result from ELISA showed that, at the D/P ratio of 2 and 3, the relative affinity of AF647-2°Mab-PC was similar to the 2°Mab-PC without conjugates (i.e., D/P=0), and at D/P ratio of 4, the relative affinity of the 2°Mab-PC to PC molecules decreased by 40%. The optimal AF647 D/P ratio for the PC sensing was, therefore, determined to be 3. The signal intensity of PC measurement using AF647-2°Mab at D/P ratio of 3 increased by approximately 115%, compared to the Cy5-2°Mab-PC at the optimal D/P ratio of 5.

In the clinical practice, a minimal bio-sample volume for an assay is desirable and, therefore, a study was performed to minimize the sensor size. The performance of a 3-cm PC sensor was tested in the target sensing range and compared with that of a 6-cm sensor (data not shown). The signal intensity of the 3-cm PC sensor also showed a linear relationship with the PC concentration, at an average signal-to-noise (S/N) ratio of approximately 25. This result demonstrated that a 3-cm PC sensor is capable of accurately quantifying PC in plasma (sample volume: ~500 μl) in the clinically important sensing range for the PC deficiency diagnosis within 5 min.

The sensing protocol of the PC sensor was further extended for the development of PS, ATIII, and PLG sensing system. The optimal flow velocity for the PS sensing was determined to be 0.5 cm/s (data not shown), which is less than the optimal velocity of the PC sensing (0.7 cm/s for 1 μg/ml PC). It is probably because the PS sensing range is higher than the PC's and therefore, less mass-transfer-limited during the reaction between PS and 1°Mab-PS, assuming that antibodies for both systems have similar affinity to respective antigens. The effects of the convective flow on the ATIII and PLG sensing performance were also studied (data not shown). Unlike the PC sensing, with a static incubation (i.e., no flow), the signal intensity was high enough to clearly differentiate ATIII or PLG concentration in the sensing range. It indicates that the diffusional mass transport is sufficient for the ATIII or PLG molecular supply to the sensor surface, since the sensing ranges are hundreds of times higher than that of PC. Similar to the PC sensor, the standard curves of the PS, ATIII, and PLG sensing were in linear relationship (r^2=0.99) with the respective analyte concentration in the sensing ranges, at a S/N ratio of 30, capable of quantifying each concentration in the blood plasma in the deficiency range within 5 min (data not shown).

3.1.2 *Simultaneous quantification of the four anticoagulants*

For the simultaneous four-anticoagulant quantification, each sensor, with the specific antibodies against respective anticoagulants immobilized on its surface, was placed in one of the four channels in the four-analyte sensing unit (Fig. 1). The unit connects the four sensors in series and when a sample flows over the four channels, each anticoagulant is captured by one of the four sensors for detection.

Cross-reactivity of the four sensors. The cross-reactivity of each anticoagulant sensor to the other three analytes was first investigated. To determine the maximum extent of the possible cross-reactivity, each sensor was tested with the other three analytes at their upper sensing limits. For example, for a PC sensor, PS (5 μg/ml), ATIII (105 μg/ml), and PLG (120 μg/ml) were tested and the fluorescent signals were compared to those of the PC measurement at 0.5 μg/ml (lower sensing limit) without other factors (Fig. 3). The responses from the PC sensor for the PS, ATIII, and PLG in the sample were less than 5% (almost not shown) of the positive response for PC at its lower sensing limit. The PS, ATIII, and PLG sensors showed similar results, indicating that all four sensors were highly specific with little cross-reactivity.

Effect of second antibody mixture on the sensing performance. For the multi-anticoagulant sensing, after the sample incubation, four different AF647-2°Mabs need to be introduced into the respective sensor chambers for quantification by fluoresence. This can be done by either applying each antibody to the respective chamber or applying a mixture of the four different AF647-2°Mabs through the four-analyte sensing unit if the mixture does not affect the sensing performance. The second case is more desirable for a multi-sensing unit because it requires an easier design for the fluid manipu-

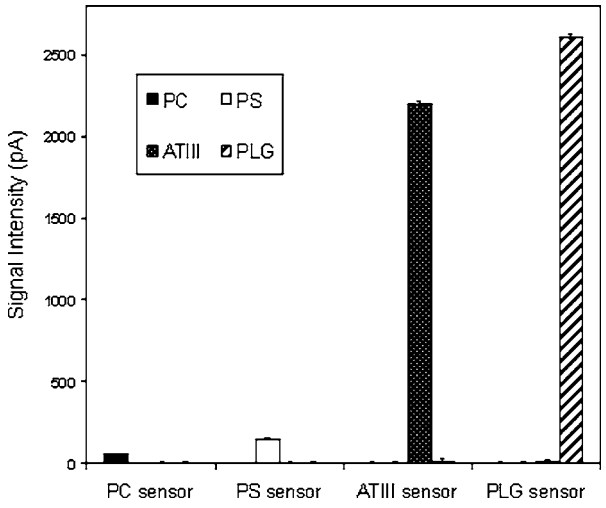

Fig. 3 Minimal cross-reactivity of PC, PS, ATIII, and PLG sensors with three other analytes (experimental conditions: 3 cm sensors; respective analyte at its lower sensing limit; the other three analytes at their upper sensing limits; incubation times for the sample and reagent were 0.5 and 2 min, respectively; flow velocity at 0.7 cm/s).

lation and automation of the sensing procedures. The effect of the AF647-2°Mabs mixture was, therefore, investigated for the sensing performance of each sensor.

Figure 4 shows the signal intensities generated by a PC sensor using only AF647-2°Mab-PC (squares) and the AF647-2°Mabs mixture (diamonds). Both measurements were performed by a single PC sensor to eliminate possible fiber-to-fiber sensing variation. The concentration of the total AF647-2°Mabs in the mixture was four times that of individual sensing. The signal intensity generated by the mixture slightly decreased, but the reduction was less than 6%, with a similar standard deviation $(5\sim10\%)$ for both measurements. The cross-reactivity among the four analytes was already proven to be minimal. The cause for the signal reduction could be

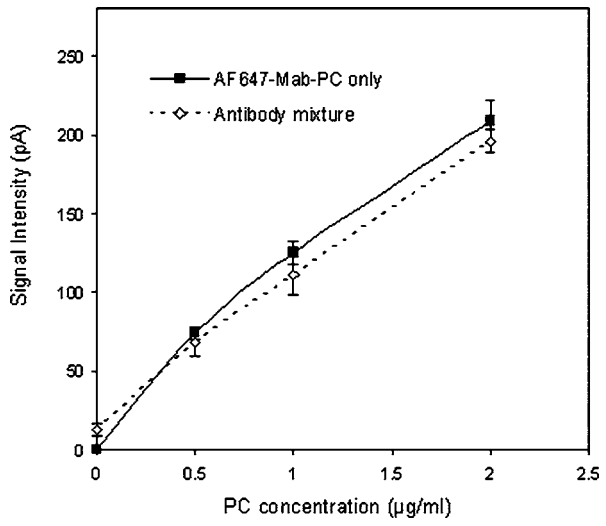

Fig. 4 Effect of the AF647-2°Mab mixture on PC sensing (experimental conditions: 3-cm PC sensor; 0.5/2 min incubation for the samples and reagents, respectively; 0.7 cm/s convective flow velocity).

that the presence of other molecules (here, AF647-2°Mab-PS, AF647-2°Mab-ATIII, and AF647-2°Mab-PLG) causes a slower mass transport rate for the AF647-2°Mab-PC in the sensing system within a limited reaction time. A more systematic investigation for the actual cause is currently ongoing. The background signal generated by the AF647-2°Mab mixture slightly increased $(10\sim15$ pA) for all the tested concentrations, compared to that by AF647-2°Mab-PC only, with a possible increase in the non-specific binding by the increase in the 2°Mab concentration.

The effect of the antibody mixture on the PS, ATIII, and PLG sensor performance was also studied (data not shown). Similar to the PC sensor, the signal intensities of the measurements with the AF647-2°Mabs mixture were decreased by less than 4.5%. For all the four sensors, the standard curves with the AF647-2°Mabs mixture were linear with the respective anticoagulant concentration ($r^2=0.99$), at an average S/N ratio of 25. These results demonstrated that the simultaneous quantification of the four anticoagulants is possible with the mixed AF647-2°Mabs with little changes in the sensitivity, especially if the standard curve is also obtained with the AF647-2°Mabs mixture. Therefore, it was decided to apply the mixture to the multi-sensing unit for simultaneous PC, PS, ATIII, and PLG detection.

Simultaneous four-factor quantification. The sensing performance of the four-analyte sensing unit for samples with PC, PS, ATIII, and PLG at their lower (0.5, 1.5, 45, and 60 μg/ml, respectively) and higher (2.5, 5, 105, and 120 μg/ml, respectively) sensing limits was first investigated (data not shown). The signal intensities of the simultaneous multi-anticoagulant sensing were decreased by 5–10% compared to those of single anticoagulant sensing at their lower sensing limits, at their upper sensing limits, approximately 3–7%. Since the specificity of the sensor to the respective analyte has been already well proven (Fig. 3), this signal reduction was probably due to the reduced diffusion of the analyte with the presence of the other analytes in the sample. This signal reduction causes little problem if the patient has only one anticoagulant deficiency because the extent of the signal reduction will be consistent. If the patient has more than one anticoagulant deficiency then it may slightly overestimate the analyte concentration in the sample. However, even with this concern, the error is less than 10%. A more systematic study is in progress to identify the actual cause for the signal reduction. Convective flow at a higher flow velocity may improve the analyte mass transport to the sensor surface to reduce the interference of the other analyte in the sample. Increased sample incubation time may also be helpful for the mass transfer in mixed sample.

Despite the slight signal reduction, the sensitivities of the four anticoagulant sensors in multi-sensing are similar to the individual anticoagulant sensing (Fig. 5). The standard curves are linear with the respective analyte concentration in the sensing ranges ($r^2=0.97\sim0.99$). The study results demonstrated that the four-analyte sensing unit is capable of performing a rapid ($\sim$5 min), accurate, and simultaneous quantification of PC, PS, ATIII, and PLG in blood plasma, at an average S/N ratio of 25.

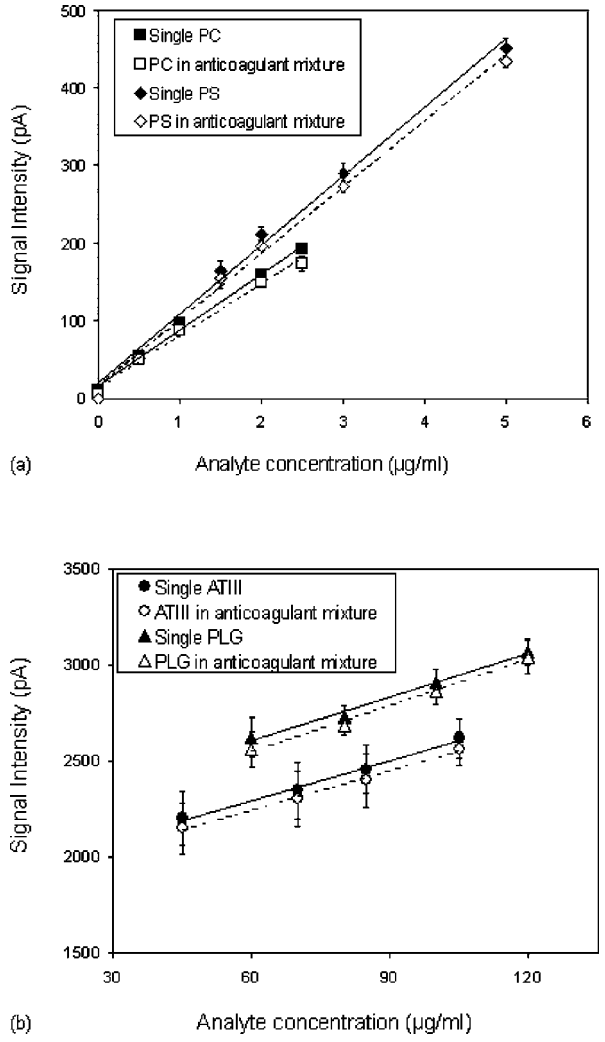

Fig. 5 Sensitivity of (a) PC (squares) and PS (diamonds), (b) ATIII (circles) and PLG (triangles) sensors in the four-analyte sensing unit for simultaneous four factor quantification. Solid lines: single anticoagulant quantification; dashed lines: anticoagulant in mixture (experimental conditions: 3-cm sensors; PC, PS, ATIII and PLG in plasma; 0.5/2 min incubation times for the sample and reagent, respectively; 0.7 cm/s convective flow velocity; mixed AF647-2°Mabs).

3.2 *Multi-Cardiac-Marker Sensing*

The fiber-optic, multi-analyte immunobiosensing system can also be applied for simultaneous quantification of multiple cardiac markers in blood plasma as a diagnostic and prognostic tool for coronary heart disease. As stated before (Table 1), the clinically significant sensing ranges for the BNP [0.1 ~ 1 ng/ml (26 ~ 260 pM)] and cTnI [0.7 ~ 7 ng/ml (30 ~ 300 pM)] are hundreds of times lower than the anticoagulants. The MG [70 ~ 700 ng/ml (4 ~ 40 nM)] and CRP [700 ~ 7000 ng/ml (5.6 ~ 56 nM)] are comparable to the anticoagulants, therefore, little difficulty was expected for the MG and CRP sensing.

Due to the extremely low target BNP and cTnI sensing range, the initial sensing study was performed with a 12-cm BNP sensor, and 10 min, each, for the sample and AF647-2°Mab incubation. With a static incubation (i.e., no flow), the signal intensity of the BNP sensing in blood plasma was only 5 ~ 10 pA. Evaluation of the Damkohler number (N_{Da}) shows that the reaction kinetics of the BNP and cTnI sensing system is mass-transfer-limited with diffusional mass transport only. To improve the sensing performance by accelerating the analyte mass transport, a convective flow was applied and the optimal flow velocity was determined to be 1.2 cm/s (data not shown). This flow velocity is much higher than the anticoagulant sensing system, because of the extremely low sensing range. The film thickness for the BNP sensing at 0.1 cm/s was calculated to be 25.9×10^{-3} cm, using the theoretical analysis by Kwon,[31] twice of that of the PC sensing system (12×10^{-3} cm).

The signal intensity for many disease representing biomarkers, such as BNP and cTnI, is extremely low due to the low target sensing range. Since the fiber-optic biosensing system is fluorophore mediated, fluorescence enhancers were applied to improve the sensor performance by enhancing the fluorescent signal. Our research group has developed novel nanometal particle reagents (NMPRs) that can improve the sensitivity of the biosensing system as high as 10 times. These reagents are specially prepared with nanometal particles in a selective, biocompatible organic solvent. The fluorescence enhancement was by (1) the transfer of the electrons, normally involved in intramolecular fluorescence quenching, from a fluorophore to a nanometal particle, via the plasmon rich, electromagnetic field surrounding the nanometal particle; and (2) the increase of the energy gap of a fluorophore between the ground and the excitation states in a solvent.[38–40] Figure 6(a) shows the standard curve of the BNP sensing with and without nanogold particle reagent (NGPR). The signal intensity increased by approximately 100–300%, with the sensor size reduced by 75% and a less than 50% of the assay time. Similar results were obtained for the cTnI, MG, and CRP sensing [Fig. 6(b)–6(d)], indicating that the 3-cm cardiac marker sensors were capable of accurately quantifying the cardiac marker concentrations in blood plasma within 10 min, at an average S/N ratio of 20.

Similar to the multi-anticoagulant sensing, the four cardiac marker sensors were placed in the four-analyte sensing unit for simultaneous multi-cardiac-marker quantification. The four cardiac markers have no known structural homology. However, to determine the possible interference to the sensing performance in the multi-sensing format, each sensor was tested with samples containing the other three analytes at their upper limit sensing ranges (data not shown), as in multi-anticoagulant sensing. The signal intensities generated by the BNP sensor for cTnI (7 ng/ml), MG (700 ng/ml), and CRP (7000 ng/ml) in the sample were less than 5% of the response for the BNP at its lower sensing limit (0.1 ng/ml). Similar results were shown for the cTnI, MG, and CRP sensors when they were probed with the other three analytes present in the sample. AF647-2°Mabs mixture was also tested and it was found that each sensor can be as accurate as that with only the respective AF647-2°Mab, with a slight signal reduction. The sensing performance of the multi-cardiac-marker quantification was comparable to the single cardiac marker sensing, at a similar S/N ratio (data not shown). Similar to the multi-anticoagulant sensing, the signal intensity for mixed sample was slightly decreased (5 ~ 10%) due to the presence of other three analytes in the sample. This prelimi-

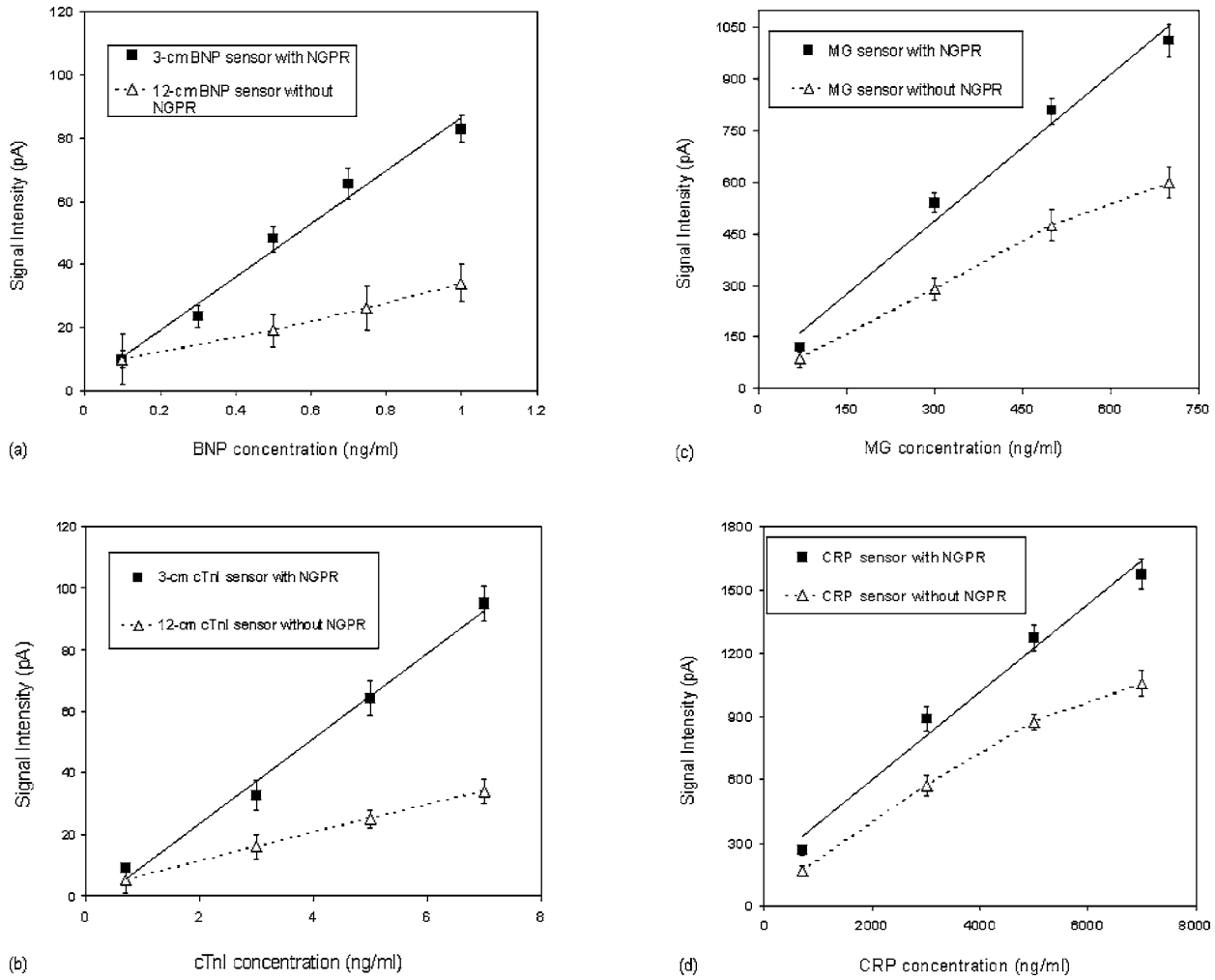

Fig. 6 Standard curves of (a) BNP, (b) cTnI, (c) MG, and (d) CRP sensing with (squares) and without (triangles) NGPR (experimental conditions: 3-cm sensors; incubation time for the sample and AF647-2°Mabs were 3 and 4 min, respectively; 1.2 cm/s convective flow velocity).

nary study result showed that the multi-sensing unit was capable of simultaneously quantifying the four cardiac markers within 10 min, at an average S/N ratio of 20.

3.3 *Automation of the Sensing Procedures with Automatic Flow Control System*

The ultimate goal of this research is to develop a rapid, automatic, and easy-to-use sensing cartridge (or chip) for CVD diagnosis and/or providing prognostic information to the surgeons at patient's bedside. The automatic operation is beneficial for a more reliable patient evaluation since the assay procedures can be well controlled with minimal human errors. As an initial effort, an automatic flow control system consisting of electrically controllable pump and valves was developed. A computer program (LabView™) electronically operates the micro peristaltic pump and six micro solenoid valves in the system. To test the sensing performance of the multi-sensing unit integrated with the automatic flow control system, a simultaneous quantification of the multi-anticoagulants was performed at their lower and upper sensing limits with manual and automatic operations. For both limits, the signal intensities of the measurements with the automatic flow control system were comparable to the manual operation.

Current effort is focused on converting this prototype system to a compact microfluidic system where all the electronic control components, micro-pump, and micro-valves are integrated on a small board inside an instrument with a space where a sensing cartridge can be inserted. The final version of the sensing system as a rapid diagnostic tool for CVD diseases is envisioned as follows: when the sample and reagent solutions are filled in appropriate reservoirs and the sensing cartridge is inserted on the instrument, an assay will be performed automatically and the quantitative results are shown in the LCD screen within 10 min with a printout.

4 Conclusions

A prototype of a semi-automatic, fluorophore mediated, fiber-optic, multi-analyte immunobiosensing system has been developed for simultaneous quantification of multiple CVD related biomarkers in blood plasma. In particular, a multi-anticoagulant and a multi-cardiac-marker sensing systems were developed for anticoagulant deficiency diagnosis and

coronary heart disease diagnosis and prognosis, respectively.

First, individual sensors (3 cm long) were developed and the sensing protocols were optimized. For simultaneous quantification of four factors in blood, the four sensors bearing antibodies specific for respective biomarkers were connected in series in a four-analyte sensing unit. The multi-anticoagulant sensing system was capable of performing a rapid (~5 min), accurate, and simultaneous quantification of PC, PS, ATIII, and PLG in blood plasma, at an average S/N ratio of 25. A simultaneous assay of the four cardiac markers (BNP, cTnI, MG, and CRP) in their clinically significant sensing ranges can be performed within 10 min, at an average S/N ratio of 20. Efforts were also made to develop an automatic flow control system to precisely and automatically perform the assay procedures for simultaneous four analytes quantification. This semi-automatic sensing system is an initial effort towards the ultimate goal of a disposable and automatic sensing cartridge as a portable diagnostic tool for CVD related diseases. This fast and portable assay system can be used not only in the emergency room for a rapid CHD diagnosis, but also for monitoring of patients at the bedside to provide prognostic information of coronary events. The principle of this multi-analyte sensing system can also be applied for multi-sensing of other disease-representing biomarkers, such as cancer markers.

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