Chapter 28
Biosensor for Diagnosing Factor V Leiden, A Single Amino Acid Mutated Abnormality of Factor V

Yongjie Ren, Samin Rezania, and Kyung A. Kang[1]

Abstract Factor V Leiden (FVL) is an abnormality with a single amino acid mutation of Factor V (FV) and is the most common, hereditary blood coagulation disorder. FVL is currently diagnosed by DNA analysis, which takes a long assay time, high cost, and a specially trained person. We are developing a rapid, accurate, and cost-effective biosensing system to quantify both FV and FVL in blood plasma, to diagnose FVL and also to evaluate the seriousness of the disease status. This system is based on a sandwich immuno-reaction on an optical fiber. To produce the monoclonal antibody against only FV or only FVL without cross-reacting with the other molecule and with a higher probability, a 20 amino acid sequence (20-mer) of FV or FVL around the mutation region was injected into mice and then hybridoma cell lines specific to each 20-mer were selected. When these antibodies were tested with native FV or FVL molecules, they were found to be cross-reacting with the other molecules, but some with higher affinity to FV (FV preferred) and some to FVL (FVL preferred). Using these antibodies, two different sensors were developed: FV preferred and FVL preferred sensors. These two sensors allowed us to quantify FV and FVL in plasma with a maximum error of 4%. The plasma levels of both molecules provide us not only FVL diagnosis but also the level of the seriousness. The same principles may be used for developing diagnostic tools for other diseases with a single point mutation.

28.1 Introduction

Factor V (FV) is an essential factor of the blood coagulation cascade [1]. Its molecular weight is 330 kDa and it is composed of a heavy chain (MW = 105 kDa) and a light chain (MW = 71∼74 kDa). When the gene for the arginine at the position 506 (R506Q) on the heavy chain of FV is mutated and replaced with glutamine, FV loses the cleavage site for activated protein C (APC;

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K.A. Kang et al. (eds.), *Oxygen Transport to Tissue XXIX*

anticoagulant) [2]. This mutation (i.e., factor V Leiden; FVL) prevents APC from the efficient inactivation of FV and facilitates overproduction of thrombin, leading to excess fibrin generation and blood clotting [3].

FVL is the most common hereditary blood coagulation disorder in the United States. It is present in 5% of the Caucasian population and 1.2% of the African American population [7]. FVL increases the risk of venous thrombosis 3–8 folds for the heterozygous and 30–140 folds for the homozygous individuals [7]. Currently, the FVL diagnosis is made by DNA analysis or by the clotting test that measures the degree of prolongation of plasma clotting time after the addition of APC [3]. DNA analysis is, however, expensive and time-consuming, and the clotting test is not specific only for FVL.

The fiber optic biosensing system is a rapid, accurate, and cost-efficient method to detect the level of the specific protein in plasma [4, 9, 10, 11]. This method performs a sandwich assay on the surface of an optical fiber.

Here, a fiber optic biosensing system for FVL diagnosis is presented.

28.2 Materials, Instruments and Methods

28.2.1 Materials and Instruments

For biosensing: The fluorometer (Analyte 2000) and the quartz fibers used for sensors were from Research International (Monroe, WA). Factor V and a monoclonal antibody against FV light chain were purchased from Haematologic Tech. (Essex Junction, VT) and another monoclonal antibody against FV light chain was from Fitzgerald (Concord, MA). The homozygous patient plasma was obtained from a FVL homozygous patient, following approval by the University of Louisville Institutional Review Board (IRB). Alexa Fluor® 647 (AF647) was purchased from Invitrogen (Carlsbad, CA). ImmunoProbe™ Biotinylation Kits, avidin, hydrofluoric acid, phosphate buffered saline (PBS), triethylamin, γ-maleimideobutyric acid N-hydroxysuccinimide ester, (3-mercaptopropyl) – trimethoxysilane were from Sigma-Aldrich (St. Louis, MO). FV free plasma was from American Diagnosica Inc. (Stamford, CT).

For enzyme linked immunoassay (ELISA): Bovine Serum Albumin (BSA) and *o*-phenylenediamine dihydrochloride (OPD) tablet were from Sigma-Aldrich. Fc specific, horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The ELISA plate reader was from Bio-Rad (Hercules, CA).

28.2.2 Methods

For biosensing by fiber optic sensing system: All sensors were prepared following the protocol established by previous researchers [4, 9, 10, 11]. Briefly, the antibody against FV or FVL (1°MAb) that we have developed using 20-mers

was immobilized on the fiber surface by the avidin-biotin linkage and then the fiber is enclosed in a sensing chamber. When a sample is injected into the chamber, the FV/FVL molecule in the sample is captured by the 1°MAb. After washing the fiber surface to remove unbound bio-molecules, the antibody against FV light chain (2°MAb) conjugated with AF647 is applied to the sensing chamber. After the sandwich complex is formed, the excitation light is applied to the sensor and the emitted fluorescence is measured by a fluorometer. The fluorescence intensity is correlated with the amount of FV/FVL in the sample. Regarding the sample and 2°MAb incubation time, for testing affinity of the generated antibodies, 10 and 10 minutes (10/10 min.) were used. For sensing analytes, 3 and 2 minutes (3/2 min.) were used.

For ELISA: To test the affinity of the antibodies generated, ELISA was performed as follows: 96 wells of an ELISA plate (Dow corning, NY) were coated with 100 μl of FV in plasma (2 μg-FV/ml-FV free plasma) or 100 μl of homozygous FVL plasma (2 μg/ml). First, the well surface was blocked with 250 μl, 1% BSA each well for 90 minutes at room temperature, then 100 μl of anti-FV antibodies (1 μg/ml) was applied on the first column wells and the ½ serial dilution was performed. After incubation at 37°C for 90 minutes, 100 μl of 1:1000 Fc specific, HRP conjugated rabbit anti-mouse IgG was applied for 20 minutes at 37°C. After washing the plate and adding 100 μl of OPD solution to each well, the plate was incubated at room temperature for 30 minutes, and then the optical density was measured at 450 nm by the ELISA reader.

28.3 Results and Discussion

28.3.1 Production of 1°MAb Against FV and FVL

Currently, neither pure FVL molecule nor the antibody against FVL without cross-reacting with FV is available. Generating antibodies specifically against a single point mutation site of a molecule is extremely difficult. To increase the probability for generating antibodies specifically against the mutation site, the 20 amino acid sequences (20-mer) of FV or FVL around the mutation site were generated (Fig. 28.1) [8]. The 20-mers were then conjugated with a carrier protein, the conjugated molecules were injected into mice, and the hybridoma cell lines were generated [9, 12]. The resulting antibodies were first screened with the 20-mers and those with high affinity to FVL molecules without cross-reacting with FV were selected, and vice versa.

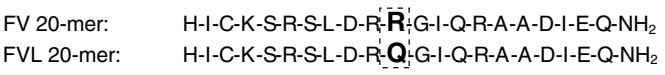

Fig. 28.1 The amino acid sequence of 20-mers for FV and FVL (the mutation sites are in bold).

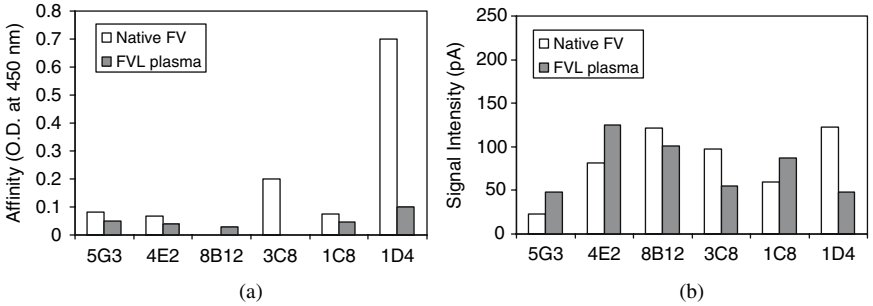

Fig. 28.2 Relative affinity values (in O.D.) of six selected antibodies against FV (□) and FVL (■) (a) by ELISA and (b) a biosensor. [Experiment conditions: 10 cm sensors, 10/10 min. sample/2°MAb incubation times, 1.2 cm/s circulation flow velocity during incubation].

The screened monoclonal antibodies were then tested with native FV molecules and homozygous FVL plasma by ELISA [Fig. 28.2(a)]. They were found to have some cross-reactivity with the other molecule. However, some had higher affinity with FV and some with FVL. The affinities of these antibodies were also tested as the 1°MAb for the sensing system, and a commercial antibody against the FV light chain was used as the 2°MAb. For the biosensor, the experiments was performed for the sample 5 μg FV/FVL in 1 ml plasma and the sensor also showed the cross-reactivity [Fig. 28.2(b)]. Interestingly, the antibody 5G3 had a higher affinity for FV in ELISA but it shows the highest relative affinity for FVL (FVL: FV = 2.17). The antibody 1D4 shows the highest relative affinity for FV (FV: FVL = 2.55), consistent with the ELISA result. Therefore, the antibody 5G3 and 1D4 are selected to be 1°MAbs for the FVL and the FV preferred sensors, respectively.

28.3.2 Sensing Performance of the FV and FVL Preferred Sensors

Heterozygous patients have both FV and FVL molecules in their blood. Quantifying both FV and FVL in their plasma provides information on the degree of the abnormality. First, the FV and the FVL preferred sensors were tested for their behaviors for FV or FVL, separately in the sample, with the physiological range of 0∼15 μg/ml-plasma (Fig. 28.3). For both FV preferred and FVL preferred sensors, the relationships between the analyte concentration and the signal intensity were linear. For the FV preferred sensor, the slope of the standard curve for FV was 9.3, which is higher than the slope of FVL 6.8, as expected [Fig. 28.3(a)]. For the FVL preferred sensor, the slopes were 8.6 for FVL and 5.4 for FV [Fig. 28.3(b)].

Next, the response of these sensors was studied for a mixture with both FV and FVL molecules. The FV preferred sensor was tested for a sample with both FV and FVL but at a constant FVL concentration (8 μg/ml) [Fig. 28.4(a)]. The

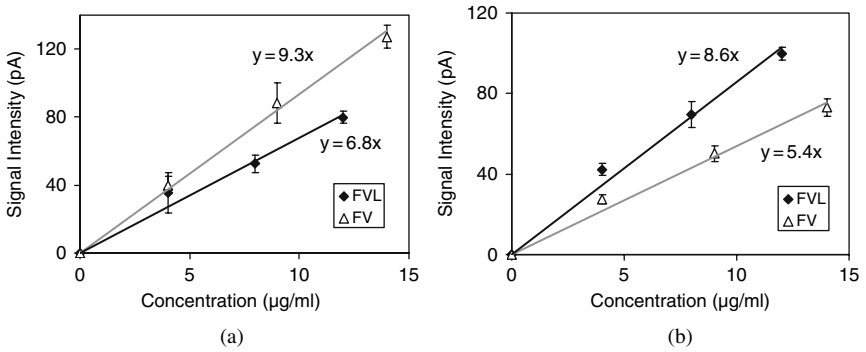

Fig. 28.3 Standard curves for FV and FVL by (a) FV preferred and (b) FVL preferred sensors. [Experiment conditions: 3 cm sensors, 3/2 min. sample/2°MAb incubation times, 1.2 cm/s circulation flow velocity during incubation].

signal intensity of the mixture was linear with the change in the FV concentration, with the slope of 8.9, which is 96% of that for FV only (9.3) [Fig. 28.3(a)]. The signal intensity of the y intercept was 54.4, the same for 8 μg/ml of FVL only. In other words, the signal intensity of the mixture was found to be the addition of the signal intensities by FV and by FVL, showing that FVL molecules in a sample do not affect the affinity of FV for the FV preferred sensor. Figure 28.4(b) confirms that FV molecules in the sample do not affect the affinity of FVL in FVL preferred sensor, either. In summary, the FV and the FVL molecules in a sample contribute to the sensor signal intensity independently, without interfering with each other.

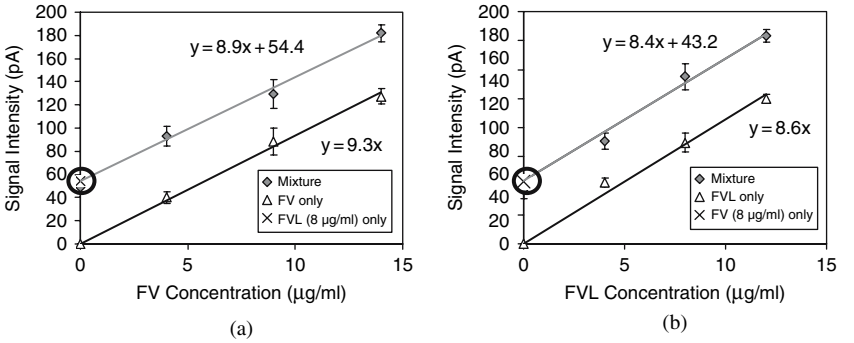

Fig. 28.4 Signal intensities of (a) the FV preferred sensor with the change in FV concentration in the sample when 8 μg/ml of FVL is added in the sample and (b) the FVL preferred sensor with the change in FVL concentration when 8 μg/ml of FV is added in the sample. [Experiment conditions: 3 cm sensors, 3/2 min. sample/2°MAb incubation times, 1.2 cm/s circulation flow velocity during incubation].

28.3.3 Quantification of FV and FVL in Blood Plasma Sample

Since our sensors may not detect FV and FVL molecules exclusively, a mathematical manipulation is needed for the quantification of these two molecules in a sample. The relationship between the two sensor signal intensities and the concentrations of FV and FVL molecules in a sample can be expressed as follows:

$$SI_1 = A_1 C_{FV} + B_1 C_{FVL} \tag{28.1}$$

$$SI_2 = A_2 C_{FV} + B_2 C_{FVL} \tag{28.2}$$

where, SI is the signal intensity (pA) generated by a sensor; A is the slope of the standard curve for FV, in pA/(μg/ml); B is the slope of the standard curve for FVL, in pA/(μg/ml); C is the concentrations of FV or FVL in the sample, in μg/ml; Subscripts 1 and 2 represent FV preferred and FVL preferred sensor, respectively. The concentrations of FV (C_{CFV}) and FVL (C_{CFVL}) can be, therefore, expressed as Eq. 28.3 and Eq. 28.4, respectively.

$$C_{FV} = \frac{\frac{SI_2}{B_2} - \frac{SI_1}{B_1}}{\frac{A_2}{B_2} - \frac{A_1}{B_1}} \tag{28.3}$$

$$C_{FVL} = \frac{\frac{SI_2}{A_2} - \frac{SI_1}{A_1}}{\frac{B_2}{A_2} - \frac{B_1}{A_1}} \tag{28.4}$$

The sensing system is composed of one FV preferred sensor and one FVL preferred sensor. A summary of the assay for FV and FVL is shown in Fig. 28.5:

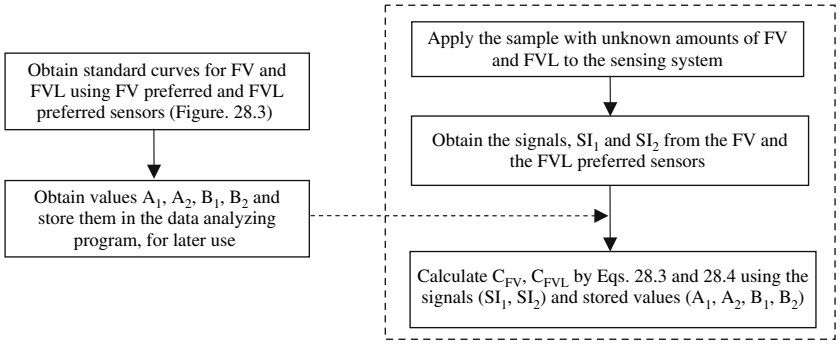

Fig. 28.5 A schematic diagram describing the procedure for obtaining the amount of FV and FVL in a sample, using our sensing system. The part within a dashed line is the actual protocol for an assay.

Table 28.1 An example of the quantification of FV and FVL in a sample. (a) the parameters of the FV/FVL sensing system and (b) the sensing result of an example

(a)

	A [pA/(μg/ml)]	B [pA/(μg/ml)]	SI (pA) from measurement #1	SI (pA) from measurement #2
FV preferred sensor	9.3	8.6	89.5	79.5
FVL preferred sensor	6.8	5.4	83.0	69.5

(b)

	Actual concentration (μg/ml)	Calculated concentration (μg/ml)	Relative error (%)
FV	5	4.8±0.1	–4
FVL	6	5.8±1.1	–3

First, the parameters need to be obtained from the standard curves of the samples of FV or FVL, and stored in the system; then the actual assay for an unknown sample will be performed as described in the block framed by a dashed line. The total assay time is about 8 minutes.

As an example, a mixture of 5 μg of FV and 6 μg of FVL in 1 ml plasma was tested by our sensing system (Table 28.1). As can be seen from the table, the FV and FVL in the sample were quantified with a relative error less than 5%.

28.4 Conclusions

The amino acid sequences of FV and FVL are different by only one amino acid. In order to increase probability for generating antibodies specific only to FV or only to FVL, 20-mers around the mutation site for each molecule were used in the hybridoma cell generation. Two antibodies with a higher affinity to FV and to FVL were selected as 1°MAb for the FV and the FVL preferred sensors, respectively. For a sample containing both FV and FVL, the total signal intensity was found to be the addition of the signals by FV and by FVL. The signals from these two sensors were used to quantify FV and FVL in a plasma sample accurately with an error of only 4%. Also, an entire assay can be completed within 10 minutes. This system is a rapid and cost-effective tool for FVL diagnosis.

The system can also be used for the diagnosis of factor V deficiency. The same principles may be applied for developing diagnostic tools for other diseases with a single point mutation.

Acknowldgment The authors thank the National Institutes of Health (5R21EB003485-02) for the financial support, and Dr. Sharma at the Hematology and Oncology Department of the University of Louisville for obtaining the plasma from a FVL homozygous patient.

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