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# Fluorescence Immunoassay of HDL and LDL Using Protein A LB Film

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**Abstract** A fluorometric detection technique for HDL (High Density Lipoprotein) and LDL (Low Density Lipoprotein) was developed for application in a fiber-optic immunosensor using a protein A Langmuir-Blodgett (LB) film. For the fluorescence immunoassay, antibodies specific to HDL or LDL were immobilized on the protein A LB film, and a fluorescence amplification method was developed to overcome their weak fluorescence. The deposition of protein A using the LB technique was monitored using a surface pressure-area ( $\pi$-A) curve, and the antibody immobilization of the protein A LB film was experimentally verified. The immobilized antibody was used to separate only HDL and LDL from a sample, then the fluorescence of the separated HDL or LDL was amplified. The amount of LDL or HDL was measured using the developed fiber optic fluorescence detection system. The optical properties resulting from the reaction of HDL or LDL with *o*-phtaldialdehyde, detection range, response time, and stability of the immunoassay were all investigated. The respective detection ranges for HDL and LDL were sufficient to diagnose the risk of coronary heart disease. The amplification step increased the sensitivity, while selective separation using the immobilized antibody led to linearity in the sensor signal. The regeneration of the antibody-immobilized substrate could produce a stable and reproducible immunosensor.

**Key words:** Coronary heart disease, fluorescence immunoassay, protein A LB film, fluorescence amplification method

Antibodies have been used extensively as diagnostic tools in many different formats [3, 7]. Today, antibody-based immunoassays are the most commonly used type of diagnostic assays and still one of the fastest growing technologies for the analysis of biomolecules [13, 14, 16]. Although antibodies are not the only type of molecule that can be used for the quantification of antigens, bioassays based on whole cells, receptors, and enzymes do not offer the same unlimited applicability and specificity in many instances.

Coronary heart disease is the major cause of death in many western industrialized countries, and, recently, has become a more important health risk in Korea. Generally, it is known that the cause of coronary heart disease is correlated with the amount of cholesterol. Also, it is thought that 'bad' cholesterol is deposited on the walls of blood vessels while it travels through the blood vessel, thereby causing serious problems in cardiovascular diseases [2, 10]. Therefore, it is important that the amount of protein transporting cholesterol is measured in order to diagnose the risk of coronary heart disease.

Two major carriers for cholesterol transportation are present in blood. It is known that one of the two carriers is deposited on artery walls, which sometimes triggers the formation of clots that block the flow of blood [2, 10]. High density lipoprotein (HDL) and low density lipoprotein (LDL) are the major carriers in blood, where the former transports 25% cholesterol in serum and the latter transports 75% [12, 18]. The level of HDL in serum is inversely proportional to the risk of coronary heart disease, whereas the amount of LDL is directly proportional to the risk of coronary heart disease [5, 9, 15]. Therefore, the respective determination of the content of HDL and LDL in serum is very important to determine the status of the risk of coronary heart disease.

A commercialized detection method of HDL and LDL has already been developed by means of immunoturbidimetry [15]. However, this detection method has several disadvantages: since an antibody is disposable due to the absence of antibody immobilization, this increases the economic cost of the detection of HDL or LDL. The detection sensitivity may be reduced by the interference of other substances in a sample as well as by the intrinsic restriction of turbidimetry. Conversely, these problems can be eliminated by antibody immobilization using the LB technique and fluorescence detection of HDL and LDL. Accordingly, the present study

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represents a new detection theory for HDL and LDL using antibody immobilization by the LB technique. In addition, a fiber-optic fluorescence detection system is also developed according to the existing detection theory.

In general, fiber-optic sensors have many advantages as follows: Fiber-optic sensors can be easily miniaturized, which can lead to the development of small, light, and flexible instrumentation. Furthermore, the measurement of different analytes can be performed in real-time, and simultaneously with the coupling of specific sensors [4]. A fiber-optic immunoassay system has a great advantage over turbidimetry, because it has a high sensitivity and accuracy. A fiber-optic assay system consists of three parts; light source, light transmission, and detection.

Since serum consists of many substances, this produces serious interference problems for target materials in fiber-optic fluorescence sensors. In the current study, the remarkable analyte specificity of an antibody is used to eliminate the background interference of unnecessary substances, select, and then separate the target organic material of HDL and LDL. The Langmuir-Blodgett technique is a very useful method for the formation of a well-ordered monolayer of organic chemicals, and ordered multilayers deposited on a substrate are also possible [4, 11]. As such, the well-defined surface of an antibody using protein A as the binding material is constructed. Protein A, a cell wall of *Staphylococcus aureus*, binds with the Fc part of the antibody [6], so that the paratope of the antibody faces the opposite side of the solid support, thereby leading to the higher efficiency of the immunoreaction and performance of the detection system. Therefore, in this study, a well-defined protein A film using the LB technique and antibody immobilization is produced under the proper deposition conditions.

Based on a preliminary study, it was found that both HDL and LDL emit weak fluorescence, which makes it impossible to detect their respective amounts in a sample. Therefore, in this study, the natural fluorescence of HDL and LDL was amplified by *o*-phtaldialdehyde to detect their respective amounts in a sample. Generally, *o*-phtaldialdehyde is used as a fluorogenic reagent for amino acids to detect proteins in a sample [17]. Finally, the optical properties of HDL and LDL, detection range, response time, and stability of the immunoassay were investigated.

## MATERIALS AND METHODS

### Preparation of Substrate

A glass plate (76 mm×26 mm, Superior, Germany) was used as the solid support for the fabrication of the protein A LB film. The glass plate was cleaned using the method as shown in Table 1, including acetone, potassium dichromate (Yakuri Pure Chemicals, Japan), and sulfuric acid (Wako Pure Chemical Industries, Ltd., Japan). The surface treatment procedure listed in Table 1 produces a hydrophilic surface and eliminates any extraneous substances on the surface. However, hydrophobic substrate is required to perform an 'x-type' deposition of protein A using a lipid monolayer. The silanization of the cleaned substrate, therefore, changed the surface property from a hydrophilic substrate to a hydrophobic surface. In order to prepare a hydrophobic surface, the substrate was dipped in a 0.2% *n*-octadecyltrichlorosilane solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 30 min. and then rinsed with fresh toluene (J.T. Baker Inc., Phillipsburg, NJ, U.S.A.) [17].

**Table 1.** Treatment procedure for optically flat polished glass slide.

| No. | Treated solvent | Treatment method |
|---|---|---|
| 1 | Acetone | Sonication |
| 2 | Distilled water | Washing |
| 3 | Distilled water | Sonication |
| 4 | Sulfuric acid+Potassium dichromate | Dipping |
| 5 | Distilled water | Washing |
| 6 | Deionized water | Sonication |
| 7 | Vacuum drying as quickly as possible | |

### Deposition of Protein A LB Film

The protein A LB film was deposited on a glass plate using a circular trough (NIMA, England). The subphase was a 1 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES, Sigma-Aldrich, St. Louis, MO, U.S.A.) buffer of pH 7.0 adjusted with 1 N NaOH (Junsei Chemical Co., Ltd., Japan) [17]. The lipids were dissolved in chloroform (Yakuri Pure Chemicals, Japan) at a concentration of 5 mM and the protein A (5 mg/vial, Sigma-Aldrich, St. Louis, MO, U.S.A. and Pharmacia, Sweden) was dissolved in 5 ml of the HEPES buffer at a concentration of 1.07 mg/l. Arachidic acid methyl ester (Sigma-Aldrich, St. Louis, MO, U.S.A.) and trimethylstearylammonium chloride (Tokyo Chemical Industries, Ltd., Japan) were used as the lipids for the protein A LB film deposition. Since arachidic acid methyl ester cannot form a monolayer by itself, it was mixed with trimethylstearylammonium chloride in a molar ratio of 1:4 [17].

The mixed lipid was then spread on a subphase of the HEPES buffer for 20 min and then compressed until the surface pressure reached to 20 mN/m (unit of surface pressure) [2]. After 5 min, a 2.5 mg/ml protein A solution was injected into the subphase to enable the protein A to be adsorbed on the spread lipid layer. The adsorption of protein A was completed after 1 h [17], which was dependent on the electrical charge difference between protein A and the lipids. Thereafter, the protein A lipid monolayer was expanded until the surface pressure became 10 mN/m, in

order that the adsorbed protein A was among the lipids [17]. Finally, the expanded monolayer was recompressed to a surface pressure of 20 mN/m so as to deposit the protein A on a glass substrate [17]. Meanwhile, the transfer was achieved with dipping conditions of 5 mm/min of downward speed and 10 mm/min of upward speed [17].

### π-A Curve

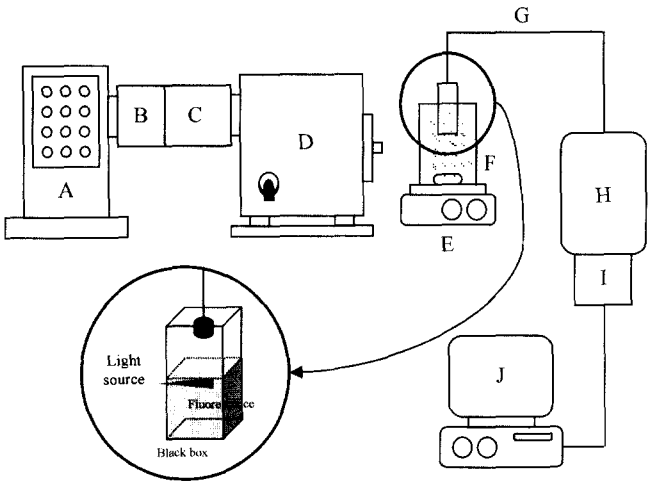
The pressure-area isotherm (π-A) is rich in information on the stability of the monolayer at the water-air interface, the reorientation of molecules in the two-dimensional system, phase transition, and conformational transformations. The π-A curve for the pure lipid monolayer was obtained using the following experimental procedures. The mixed lipid was spread on a subphase of the HEPES buffer for 20 min, and then compressed until the monolayer collapsed. The π-A curve for the lipid-protein A layer was obtained using the following experimental procedures. The mixed lipid was spread on a subphase of the HEPES buffer for 20 min and then compressed until the surface pressure reached to 20 mN/m [17]. After 4 min, a 2.5 mg/ml protein A solution was injected into the subphase to allow the protein A to be adsorbed on the spread lipid layer. Another 5 min later, the lipid-protein A monolayer was expanded until the surface area reached the initial area. Finally, the layer was compressed until it collapsed.

### Immobilization of Antibody

The polyclonal anti-HDL and polyclonal anti-LDL antibodies (SIGMA, St. Louis, MO, U.S.A.) were used respectively to recognize HDL (cholesterol: >25 μg per mg of protein, SIGMA, St. Louis, MO, U.S.A.) and LDL (cholesterol: >500 μg per mg of protein, SIGMA, St. Louis, MO, U.S.A.) in a sample. To immobilize these two antibodies on the protein A film, the film was dipped into an antibody solution (the concentration of the antibody solution was predetermined by SIGMA) for 12 h, and-the antibody-immobilized substrate was then rinsed with deionized water for 2 min.

### Fiber-Optic Fluorescence Detection System

Figure 1 shows the proposed fiber-optic fluorescence sensors composed of three parts: the light source (150 W Xenon lamp), light transmitter (Optical fiber), and light detection (Spectrograph and Photodiode array), which were purchased from ORIEL Instruments (Stratford, CT, U.S.A.). As an additional part, a rectangular reactor with set holders for the antibody-immobilized substrate was also designed. Since light is used as the detection means in fiber-optic sensors, certain optical interferences can cause serious problems, such as light scattering, etc. Therefore, the reactor was coated with a nonfluorescent material to eliminate any background interferences due to ambient light.




A. Xenon lamp (150 W)
B. Focusing assembly
C. Light shield
D. Monochromator
E. Magnetic stirrer
F. Detection vessel
G. Optical fiber
H. Spectrograph
I. Multispec II (Analyzer)
J. IBM compatible PC

**Fig. 1.** Schematic diagram of the fiber-optic fluorescence detection system.

### Immunoreaction and Fluorescence Amplification

The immunoreaction of the immobilized antibodies and antigens (HDL, LDL) was accomplished by dipping the antibody-immobilized substrate in a sample for 10 min and 6 min, respectively. One-half ml of *o*-phtaldialdehyde and 0.5 ml of 2-mercaptoethanol were added into the buffer containing the separated HDL and LDL to amplify their fluorescence. Both reagents were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). After reacting for 5 min, a fluorophore emitting strong fluorescence was produced.

### Detachment of HDL and LDL from the Immobilized Antibody

Before the fluorescence amplification step, only HDL or LDL was separated from the immobilized antibody by a detachment step in a pH 2.4 glycine buffer (1 M, 15 ml, glycine: Sigma-Aldrich, St. Louis, MO, U.S.A.) for 5 min, and then the antibody-immobilized substrate was removed from the buffer.

### Fluorescence Detection

The chemically treated samples were then ready for fluorescence detection using the proposed fiber-optic detection system. The fluorescence detection was performed with an IBM-compatible PC. To perform the data acquisition, a control program purchased from Oriel Instruments (Stratford, CT, U.S.A.) was used to control the physical detection conditions, such as the detection mode, exposure time, and so on. The typical settings for the data acquisition

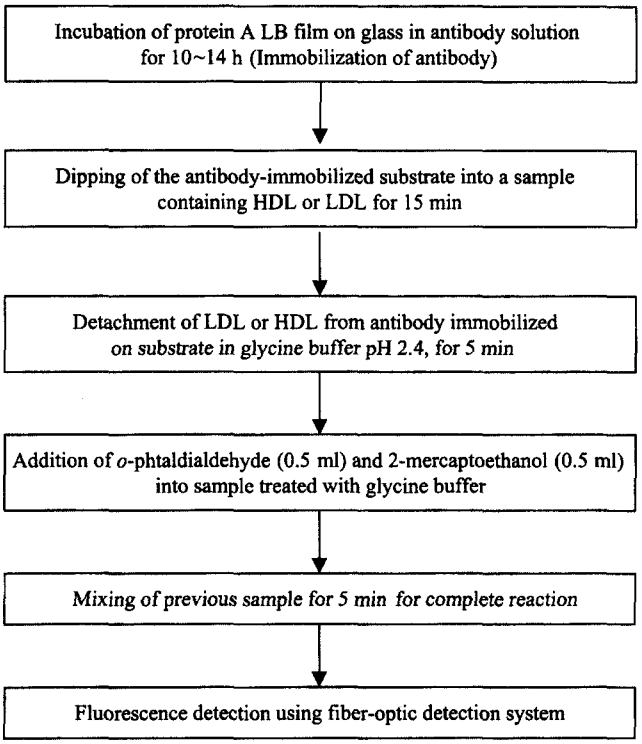



**Fig. 2.** Experimental procedure of fluorescence immunoassay of HDL or LDL.

are given below. The raw fluorescence data was manipulated using sorting, filtering, and averaging programs. The detection conditions were as follows: Detection mode: integration mode, Exposure time for 3 sec; No. of integrations, 50. The experimental procedure for the fluorescence immunoassay of HDL and LDL is shown in Fig. 2.

## RESULTS AND DISCUSSION

### Deposition of Protein A LB Film: π-A Isotherm

A monolayer of protein A molecules was expected to be formed by the lipid layer with a positive charge in the buffer solution at pH 7.0, because of the negative charge of protein A. The formation of such a lipid protein A monolayer was experimentally proved from the π-A curve, as shown in Fig. 3. The π-A curve for the lipid monolayer, after protein A adsorption, shifted within a range of 30–80 $cm^2$ in comparison with that of the lipid monolayer before protein A adsorption. It is thought that the shifting of the π-A curve for the lipid layer after protein A adsorption resulted from an increase in the number of molecules per area. In other words, the increase in the surface pressure implied that the number of molecules occupying the constant area was increased by protein A adsorption. Therefore, it was concluded that the protein A monolayer was formed by the lipid monolayer

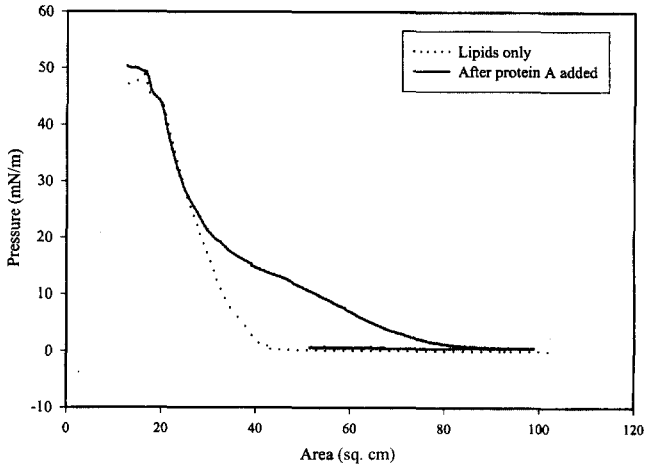



**Fig. 3.** π-A Curve of lipid layer and protein A lipid layer. Symbols: lipid layer (solid line); protein A and lipid layer (dashed line).

and, therefore, could be deposited onto a hydrophobic substrate.

### Fluorescence Amplification and Detection

It has already been reported that several fluorophores (Excitation [Ex.]/Emission [Em.]: 370/470 nm, 355/430 nm, 355/470 nm, and 380/540 nm) are present in the lipid and protein fraction LDL [1, 8]. Accordingly, the fluorescence profile of LDL was investigated using the proposed fiber-optic fluorescence system and the results are shown in Fig. 4. Similarly, HDL was also expected to include certain fluorophores with emission peaks of 290 and 440 nm. However, since both HDL and LDL have a weak fluorescence, it was difficult to measure their respective levels based on just their fluorescence with the proposed detection system. Therefore, a fluorescence amplification

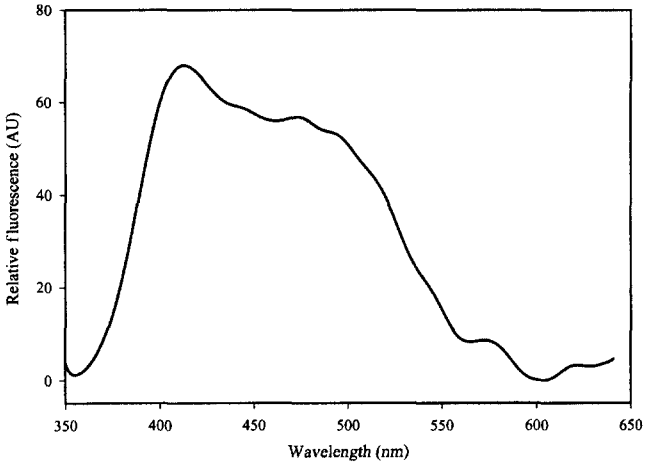



**Fig. 4.** Fluorescence profile of LDL and excitation wavelength of 355 nm (110 mg/dl LDL, using phosphate buffer of 10 mmol/l, pH 7.4, 160 mmol/l NaCl).

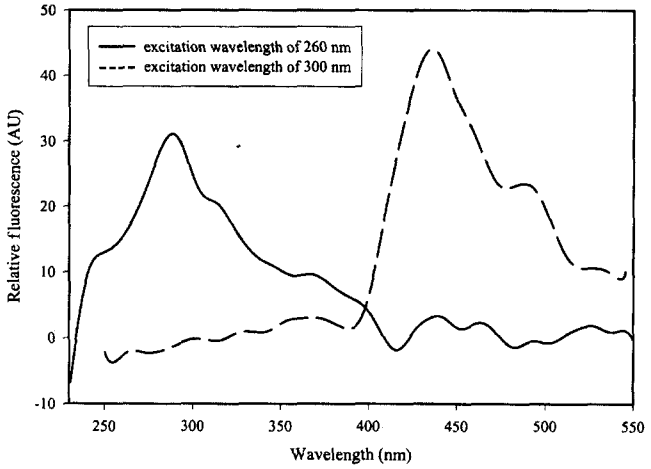



**Fig. 5.** Fluorescence profile of HDL at excitation wavelength of 260 nm and 300 nm (130 ng/dl LDL, using phosphate buffer of 10 mmol/l, pH 7.4, 160 mmol NaCl).
Symbols: Ex. 260 nm (solid line); Ex. 300 nm (dashed line).

procedure was included in the treatment of the samples, which improved the detection performance.

The respective fluorescence signals of HDL and LDL were amplified using a reagent for amino acids, *o*-phtaldialdehyde, which has been used as a fluorogenic reagent for amino acids in order to detect proteins [17]. The *o*-phataldialdehyde reacted with HDL or LDL to produce a new fluorophore emitting a strong fluorescence, which compensated for the weak fluorescence of 455 nm with an excitation of 340 nm. The HDL and LDL treated with *o*-phtalidaldehyde emitted a much stronger fluorescence than their own natural fluorescence by themselves. In addition, the S/N (signal to noise) ratio was reduced so that the sensitivity of the immunosensing system could also be improved.

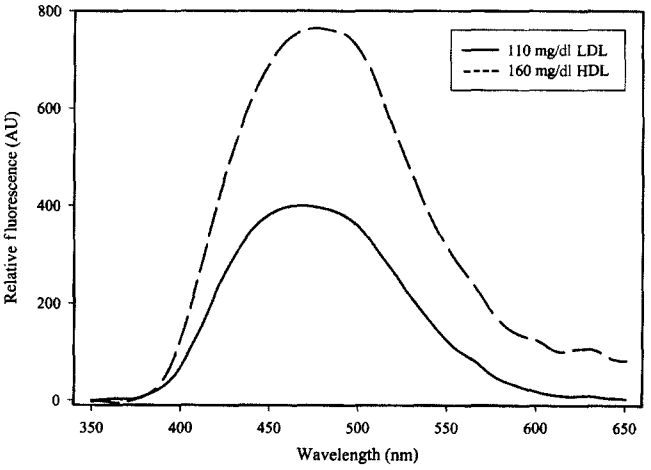



**Fig. 6.** Amplified fluorescence of HDL and LDL (at glycine buffer, 1 M, pH 7.2).
Symbols: 110 mg/dl LDL (solid line); 160 mg/dl (dashed line).

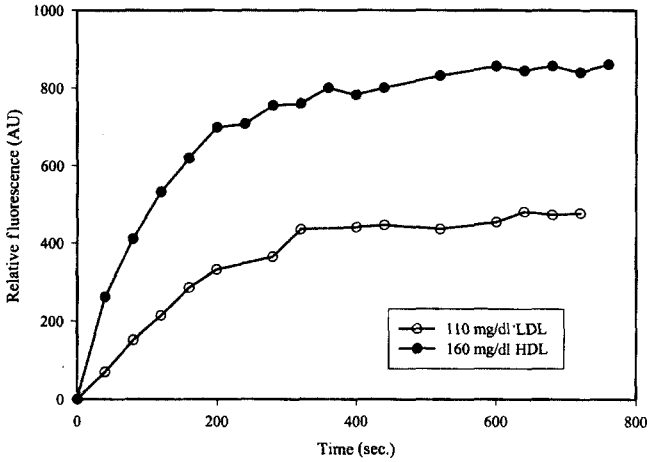



**Fig. 7.** Transient response of amplification reaction of HDL and LDL (at glycine buffer, 1 M, pH 7.2).
Symbols: 160 mg/dl HDL (●); 110 mg/dl LDL (○).

The transient fluorescence response was applied to determine the complete reaction time for the amplification of HDL and LDL. The respective transient responses for HDL and LDL are shown in Fig. 7. Data on the transient responses indicated that the reaction between either HDL or LDL and the fluorogenic reaction of *o*-phataldialdehyde was completed within 5 min.

### Immunoreaction Time and Fluorescence Detection

The transient fluorescence detection of HDL and LDL was applied to determine the immunoreaction between the immobilized antibody and the antigen, as shown in Fig. 8. HDL and LDL reacted with the immobilized antibody for 15 min. The immunoreaction time in the bulk phase [15] and on the LB film are shown in Table 2. The fast reaction on LB film resulted from the effective orientation of the

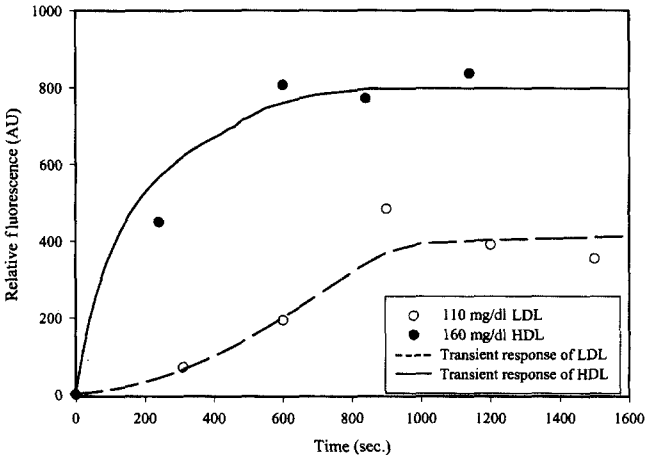



**Fig. 8.** Transient response of immunoreaction of HDL or LDL and immobilized antibody (at glycine buffer, 1 M, pH 7.2).
Symbols: 160 mg/dl HDL (●); 110 mg/dl LDL (○).

**Table 2.** Immunoreaction time on solid support and in bulk phase.

| | On solid support | In bulk phase |
|---|---|---|
| HDL | 10 | 15 |
| LDL | 15 | 6 |

immobilized antibody. The reaction time for HDL on the LB film was the same as that in the bulk phase, whereas the reaction time for LDL was a little longer than that in the bulk phase. The larger molecular size of LDL compared to that of HDL may have affected the reaction time on the solid support.

## Calibration Curve

Figure 9 shows that the calibration curves for HDL and LDL had a good linearity in a detection range of 40–230 mg/dl for HDL and 20–200 mg/dl for LDL. The detection ranges for HDL and LDL were sufficient to diagnose the risk of coronary heart disease with regards to their level in a patient, that is about 98 mg/dl and 114 mg/dl, respectively [9]. When comparing the slopes of the calibration curves, the detection sensitivity for HDL was higher than that for LDL. This difference in detection sensitivity might have been due to an affinity difference between the antibodies. Generally, detection samples should be sufficiently diluted to reduce any background interference from other materials in the sample [4]. However, in this case, no dilution step was required, because the specificity of the antibody effectively eliminated the background signals of all other materials in the fluorescence detection.

## Recycling of Antibody-Immobilized Substrate

To investigate the recycling of the antibody-immobilized substrate, the antibody-immobilized substrate was reused

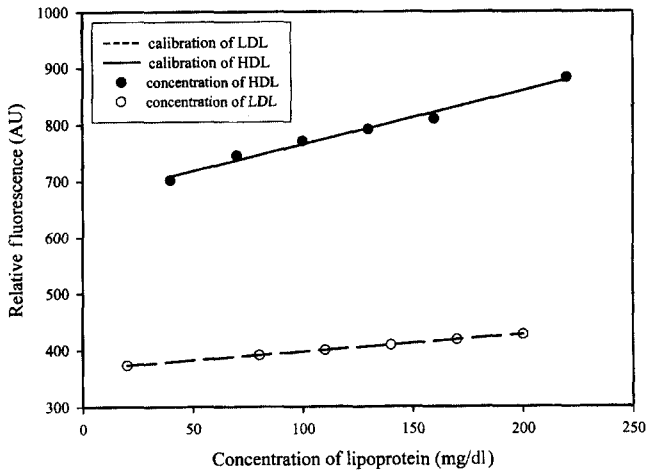



**Fig. 9.** Calibration curve of HDL and LDL.
Symbols: concentration of HDL (●); concentration of LDL (○).

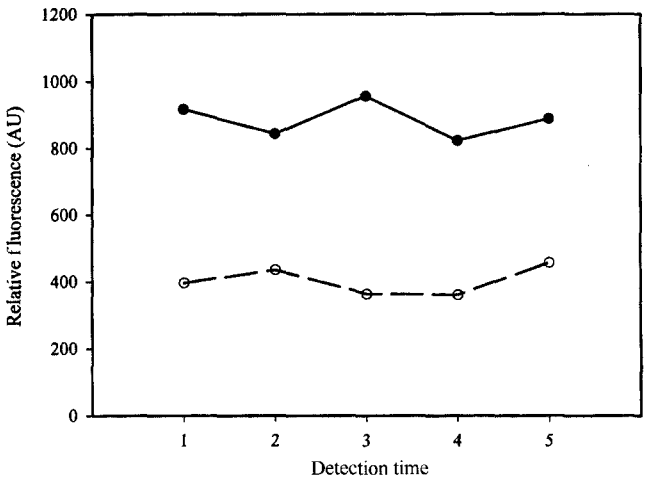



**Fig. 10.** Recycling of antibody-immobilized substrate (using glycine buffer of 1 M, pH 7.2).
Symbols: 160 mg/dl HDL (●); 110 mg/dl LDL (○).

for the fluorescence detection of HDL and LDL. As shown in Fig. 10, the antibody-immobilized substrate could be recycled at least 5 times without any activity loss. This result would be of great interest in the application of biomaterial-immobilized substrates for chemical or optical biosensors. Although the antibodies used in the current study were very expensive in contrast to conventional disposable immunoassays, the recycling of an antibody-immobilized substrate could effectively decrease the cost of detecting of HDL and LDL.

## Effect of Buffer pH on Fluorescence

The effect of the pH in the buffer solution on the fluorescence emission was investigated when detecting the levels of HDL or LDL. The pH of the glycine buffer solution was found to affect the fluorescence intensity, as shown in Fig. 11. The fluorophore in glycine buffer

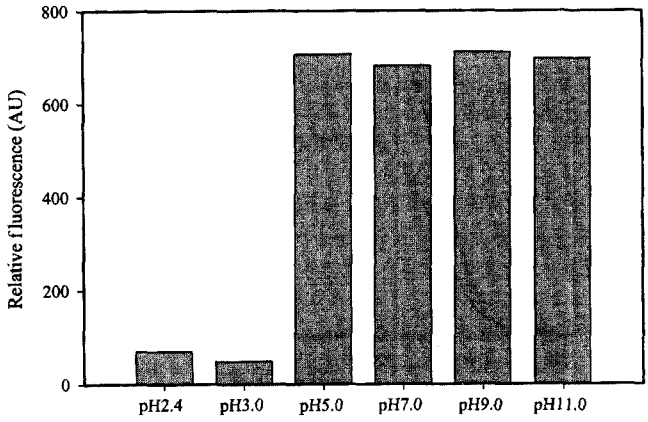



**Fig. 11.** Effect of buffer pH on fluorescence (40 mg/dl HDL, using glycine buffer of 1 M).

solution emitted fluorescence three-folds stronger than that in the phosphate buffer (data not shown). However, only a low pH below 5.0 had a significant influence on the fluorescence emission.

The proposed fluorescence immunoassay system showed that the fabricated protein A LB film was successfully immobilized using lipid, and the antibodies bound on the LB film was stable as proved in the recycling experiment. The fabricated stable protein A LB film will be a versatile tool for many kinds of immunoassay, and possibly to construct a protein chip.

## Acknowledgment

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