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On-Site Diagnostic Device Based on Immuno-Separation of Proteins

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Abstract–A membrane immuno-chromatographic system that selectively separates plasma lipoproteins and generates a signal in proportion to the concentration of cholesterol (HDL-C) within high-density lipoprotein (HDL) was investigated as a point-of-care device for the prognosis of coronary heart disease. The system consists of three functional membrane strip pads connected in a sequence for: (from the bottom) immuno-separation based on biotin-streptavidin reaction, catalytic conversion of cholesterol to hydrogen peroxide, and production of a signal. For immuno-chromatography, a monoclonal antibody, specific to apolipoprotein B100 that is present on the surfaces of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL), with a high binding constant (5×10^{10} L/mol) was raised and chemically conjugated to streptavidin. The conjugate was first reacted with lipoprotein particles, and this mixture was absorbed by the capillary action into the biotin pad of the system. After being transferred by medium, immuno-capture of LDL and VLDL particles onto the biotin pad took place, and *in situ* generation of a signal in proportion to HDL-C consecutively occurred. The capture was selective as well as effective (minimum 90% of LDL and VLDL in clinical concentration ranges), and the detection limit of HDL-C was far lower than 20 mg/dL. To construct a user-friendly device, we are currently investigating the automation of such processes of reactions and separation by adapting a liquid flow-controlling technology that programs the times for the immune reaction and separation. My group further pursues an interdisciplinary study to develop a micro system employing semiconductor-based technologies that will eventually enable the handling of sub-micro liter volume of body fluid as a specimen.

Key words: Point-of-Care-Testing, Membrane Strip, Immuno-Chromatography, Plasma Lipoprotein Cholesterol, Non-Invasive Diagnosis

INTRODUCTION

A complete separation of a particular protein even on a micro scale can be accomplished by utilizing antigen-antibody reactions in a solid phase. Since an antibody can recognize only one substance (i.e., antigen) or few compounds having structures similar to the antigen, immuno-separation employing a highly specific reaction usually yields an ultra-pure product of the process. A competing technology to such performance as to the isolation of protein has not yet emerged to date. My research group has concentrated on the development of various diagnostic systems based on immuno-separation utilizing a membrane strip as solid matrix for the immobilization of antibodies. A typical example among the outcomes is the development of a point-of-care device quantifying cholesterol (HDL-C) within plasma high-density lipoprotein (HDL) as an indicator of the incidence of coronary heart disease (CHD).

Cholesterol is an essential material in the human body: for example, a precursor of steroid hormone and a constituent of cell membranes. The lipophilic substance is transported between peripheral tissues and the liver by plasma lipoproteins [Gotto et al., 1986; Johnson et al., 1991; Mackness and Durrington, 1992; Noble, 1995]. These have the structure of a spherical particle containing esterified cholesterol and triglyceride within a surface layer consisting of phospholipid, free cholesterol, and embedded proteins (apolipoproteins). Three major classes of the lipoproteins, mainly classified according to their densities, are very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) [Bachorik and Albers, 1986; Bachorik and Ross, 1995; Nauck et al., 1995; Rudel et al., 1974].

LDL is a relatively cholesterol-enriched product converted via the lipolysis of VLDL that is synthesized in the liver [Mackness and Durrington, 1992], and transports cholesterol to tissues. HDL produced in the liver and small intestine [Tall, 1990] reversely transports cholesterol from tissues to the liver for elimination [Gordon and Rifkind, 1989; Johnson et al., 1991; Mackness and Durrington, 1992; Tall, 1990]. An increase of LDL cholesterol (LDL-C) level in blood may result in the deposition of cholesterol molecules in the arterial wall, which could eventually lead to CHD [Bachorik and Ross, 1995; Rifai et al., 1992]. On the other hand, the level of HDL cholesterol (HDL-C) is inversely correlated with the incidence of CHD [Gordon and Rifkind, 1989; Gordon et al., 1977; Johnson et al., 1991; Tall, 1990]. It has been reported that decreased HDL-C as well as increased LDL-C are independent factors causing CHD [Schaefer, 1991; Warnick and Wood, 1995].

We have investigated a novel analytical system that can be used for a routine monitoring of CHD risk. The system can measure a single-class plasma lipoprotein cholesterol, such as HDL-C and LDL-C, by means of immuno-chromatography on membrane strip and *in situ* signal generation. In this communication, the membrane strip system using a lateral flow technology is reviewed, and an automation of the analytical processes and scale-down approach to bio-

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micro system are discussed.

MATERIALS AND METHODS

1. Materials

Human lipoproteins, apolipoproteins, enzymes, and streptavidin (SA) were purchased from Calbiochem (La Jolla, CA). Cholesterol calibrators, sodium cholate, 3,3'-diaminobenzidine (DAB), poly-L-lysine hydrobromide (PLL), bovine serum albumin (BSA), and chromatographic gels were obtained from Sigma Chemical Co. (St. Louis, MO). Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin), sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-caboxylate (SMCC), dithiothreitol (DTT), and N-succinimidyl-3-[2-pyridyldithil] propionate (SPDP) were products of Pierce (Rockford, IL). Nitrocellulose (NC) membranes and cellulose membranes were purchased from Millipore Corp. (Bedford, MA) and Whatman Ltd. (Singapore), respectively. All other reagents used were of analytical grades.

2. Monoclonal Antibody

Monoclonal antibody to apo B100 was produced from BALB/c mice by following a standard method [Kohler and Milstein, 1975; Paek et al., 1993]. Tests for screening were performed by utilizing human plasma lipoproteins (VLDL, LDL, or HDL) as described elsewhere [Paek et al., 1993]. Finally, the antibody was purified on Protein G column [Hermanson et al., 1992].

3. Signal Generation

For the generation of a signal proportional to total cholesterol level in a sample, an assay system was constructed by integrating the following three membrane strip pads (see Fig. 1): sample application pad (in place of antibody pad), enzyme pad, and signal pad [Paek et al., 1999]. The pads prepared were partially superimposed on each other in an arrangement and mounted onto a polyester film by using double-sided tape. Such devised strip systems were used for assays of lipoprotein cholesterol and the signals generated after 10 min were measured by a method of scanning photometry [Paek et al., 1999].

4. Immuno-Separation of Lipoproteins

To measure a single-plasma lipoprotein cholesterol, the strip system used for measuring total cholesterol was supplemented by introducing the monoclonal antibody to apo B100 raised as described above. An antibody pad was prepared by immobilizing the monoclonal antibody on an NC membrane via physical adsorption or chemical linkage [Paek et al., 1999]. A biotin pad was also fabricated by chemically reacting biotin-BSA conjugates to the NC membrane. As another component, antibody-SA conjugates were synthesized by reacting SMCC-modified antibody with SPDP-activated SA after treatment with DTT [Paek et al., 1999]. A membrane strip system incorporated with either the antibody pad (refer to Fig. 1) or biotin pad (Fig. 7) was tested to select an effective method for immuno-separation of lipoprotein particles as previously described [Paek et al., 1999].

RESULTS AND DISCUSSION

1. Analytical Concept

A membrane immuno-chromatographic procedure may offer a simultaneous operation of the two processes, i.e., immuno-separation of a single plasma lipoprotein and enzymatic conversion of cholesterol within the lipoprotein particles. An assay system that can provide such performance consists of three major components (Fig. 1; HDL-C used as analyte in the model system): 1) antibody pad with an antibody, specific to apolipoprotein (apo) B100 that is present on the surfaces of LDL and VLDL particles [Fisher and Schumaker, 1986; Mackness and Durrington, 1992], immobilized on the surfaces of NC membrane, 2) enzyme pad with reagents decomposing lipoproteins and cholesterol, i.e., a detergent, two enzymes, cholesterol esterase (CE) and cholesterol oxidase (CO), and a chromogenic substrate for horseradish peroxidase (HRP), impregnated in a cellulose membrane, and 3) signal pad with HRP immobilized on a defined region of NC. To construct an analytical system, the membrane pads are partially superimposed and then attached to a backing support (refer to Fig. 1).

Material transfer invoked by wicking through membrane pores of the system serially connects different reaction processes taking place on each functional pad without handling reagents (Fig. 1). A sample containing mixed plasma lipoproteins is absorbed from the

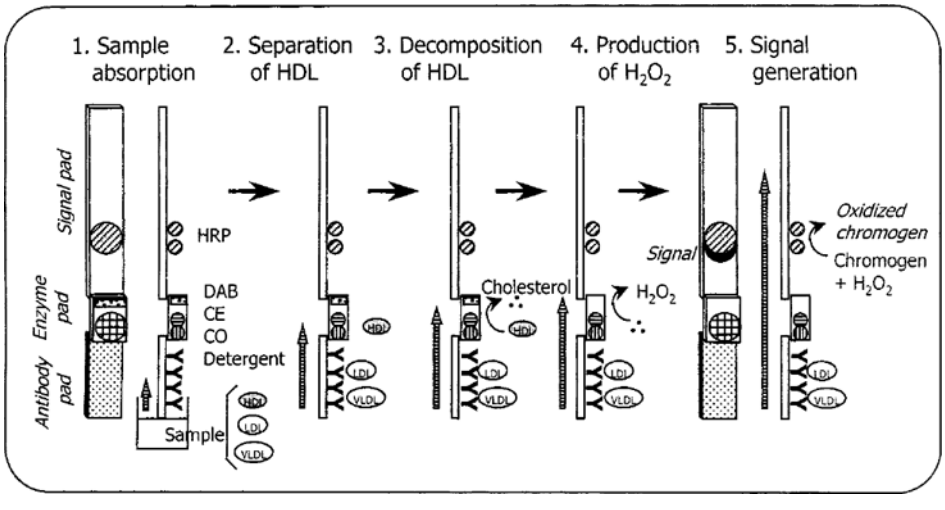

Fig. 1. The analytical concept of an immuno-chromatographic assay system that measures the concentration of a single-class lipoprotein cholesterol, exemplified by the measurement of HDL-C.

bottom of the membrane strip system (Fig. 1.1). The aqueous solution is transported upward by capillary action along the antibody pad, and two lipoproteins, LDL and VLDL, bind to the immobilized antibody while HDL is carried by medium into the enzyme pad (Fig. 1.2). The HDL particles are destroyed in the presence of detergent, and cholesterol and cholesteryl ester are then released (Fig. 1.3) to decompose and produce hydrogen peroxide by employing the following sequential enzyme reactions (Fig. 1.4) [Artiss et al., 1991; Mackness and Durrington, 1992]:

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\text{Cholesterol esterase (CE)}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase (CO)}} \text{Cholesten-4-one} + H_2O_2$$

Upon transfer into the next pad, hydrogen peroxide sequentially reacts with enzyme HRP in the presence of chromogen as follows:

$$H_2O_2 + \text{Chromogen} \xrightarrow{\text{Horseradish peroxidase (HRP)}} \text{Oxidized chromogen} + 2H_2O$$

Such a series of reactions produces a color from the oxidized chromogen as a signal on the membrane surface (Fig. 1.5). The color can be readily measured by converting it to optical density [Nilsson et al., 1995; Sterling et al., 1992] in proportion to the concentration of HDL-C. The analysis can be completed without adding or removing reagents and also within a relatively short time (e.g., 10 minutes).

2. Signal Generation

In order to facilitate the quantitative evaluation of the immunoseparation that is the key process in the cholesterol analysis, we first optimized a system for signal generation [Paek et al., 1993]. The enzyme pad of the system was fabricated by modifying previous investigations for total cholesterol [Allen et al., 1990; Law et al., 1997; Noble, 1933]. The enzyme pad contained a detergent (e.g., sodium cholate and Triton X-100) for disintegrating lipoprotein particles, two enzymes CE and CO for decomposing cholesterol, and a chromogenic substrate (e.g., DAB) for HRP [Hsu and Soban, 1982; Paek et al., 1999] located on the signal pad. The color signal converted from DAB was further intensified by introducing two metals, cobalt and nickel [Johnson et al., 1991]. These components were deposited within interstitial spaces of cellulose membrane (Whatman Qualitative #1) that were also used for enzyme reactions during assay. The relative concentration of CE to CO was determined to obtain a broad dynamic range in the dose-response curve.

The signal generation system fabricated under optimal conditions [Paek et al., 1993] was used to obtain a dose-response curve to standard concentrations of lipoprotein cholesterol (Fig. 2). The signal expressed in optical density varied in a range of 20 to 200 mg/dL cholesterol in proportion to the concentration. Since the color intensity at 20 mg/dL cholesterol was significantly higher than the background, i.e., signal at zero dose, the detection limit of the system was determined to be far lower than the concentration. In most clinical samples, the cholesterol levels of each plasma lipoprotein distribute between 20 and 70 mg/dL for HDL-C and 100 and 200 mg/dL for LDL-C [Mackness and Durrington, 1992; Schaefer, 1991]. Therefore, the enzyme pad system devised can be used to quantitatively assess the risk factors of CHD.

3. Immuno-Separation of Lipoproteins

3-1. Apolipoproteins as Specific Markers

The concentration of cholesterol in a single-class of lipoprotein (HDL in the model system) can be measured after separation of the other lipoproteins by using antigen-antibody reactions in a solid phase (see Fig. 1). As shown in Fig. 3, the plasma lipoproteins have the shape of a spherical particle containing protein(s), i.e., apolipoprotein (in abbreviation, apo), embedded on the phospholipid membrane surface. Each lipoprotein holds different profiles of protein (refer to the table in Fig. 3), which enables us to isolate a single species provided antibodies specific to apolipoproteins present exclusively on the other lipoprotein particles are used for the immobilization. For instance, if an antibody to apo B-100 contained in LDL and VLDL was placed on the antibody pad as demonstrated in Fig. 1, HDL can be separated for quantifying its cholesterol concentration. Likewise, the utilization of antibodies to the other apolipoproteins such as apo A, C, and E permits the fractionation of LDL.

3-2. Immune Reactions at Liquid-Solid Interface

In most immune reactions at liquid-solid interface, the yield of complex formation mainly depends on the binding constant of the antibody and the accessibility of antigen to the immobilized antibody [Schramm and Paek, 1992]. Based on preliminary experimental results, we selected a monoclonal antibody, specific to apo B100, with the highest binding constant of 5×10^{10} L/mol among the available. Under this condition, the accessibility of antigen may limit the binding complex formation and, thus, we have concentrated on the provision of a better accessibility of the lipoprotein particles to the binding sites of the insolubilized immunoglobulins.

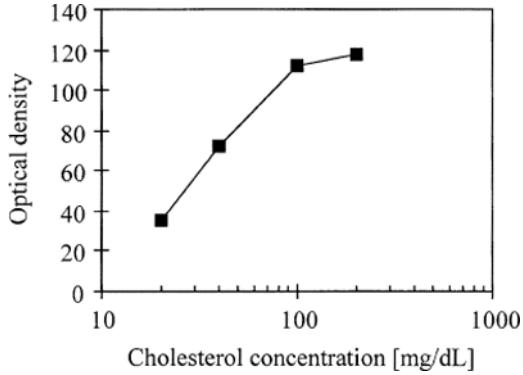

Fig. 2. Dose-response curve from the membrane strip system to standard concentrations of lipoprotein cholesterol.

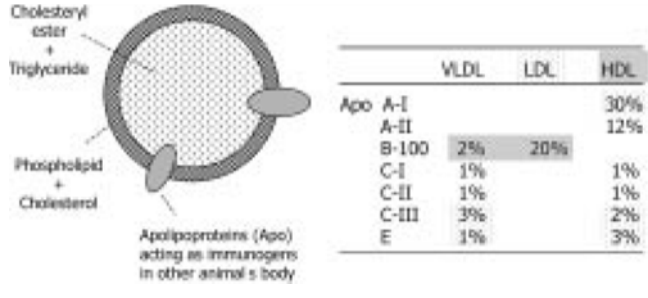

Fig. 3. The structure of plasma lipoproteins (left) and distribution of apo (lipoproteins) on the particles in different classes of lipoproteins.

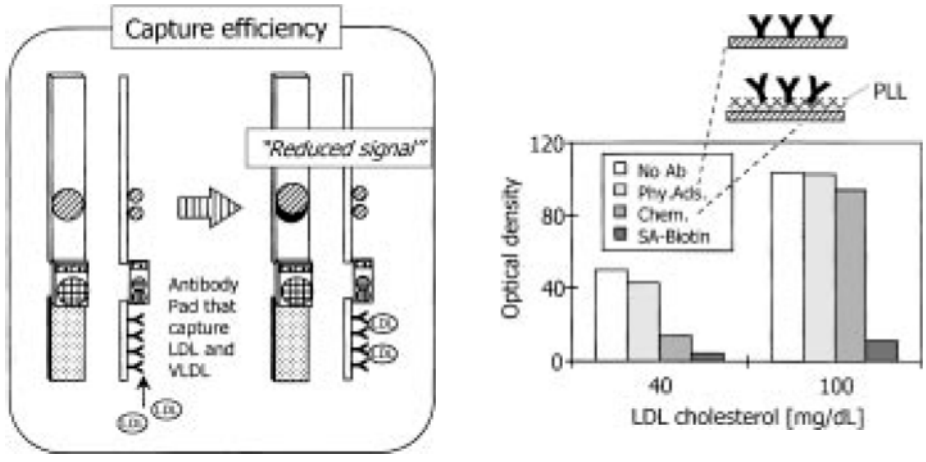

Fig. 4. Comparison of the efficiencies of immuno-separation of LDL with an antibody immobilized on the membrane surfaces of the antibody pad by using variable methods. See text for details.

The accessibility highly depends on the size of antigen and immobilization method of antibody [Cantarero et al., 1980; Matson and Little, 1988; Schramm and Paek, 1992]. The size of LDL and VLDL particles, for example, is distributed in a range of 19 to 45 nm in diameter [Mackness and Durrington, 1992], which is typically 10 times larger (i.e., 1000 times larger in volume) than those of other proteins. For a better complex formation at the interface, different methods of immobilization were attempted for comparison (Fig. 4). A sample containing LDL was added to the analytical system shown in Fig. 1, and the binding efficiency onto the antibody pad was determined by measuring the decrease of signal as compared to the control obtained in the absence of antibody (No antibody, Fig. 4). If the antibody was immobilized by either physical adsorption (Phy. Ads.) or chemical binding via polymer strands of PLL onto the pad (Chemical), the degree of signal reduction was insignificant or not sufficient for the capture of a large quantity of LDL (e.g., 100 mg/dL). In addition, although we have also increased the surface density of immunoglobulins by adding a high concentration of antibody or introducing a small pore size of membrane, no profound improvement in the capture efficiency was obtained (data not shown).

3. Limiting Factors of the Accessibility

Immunoglobulin is a relatively huge Y-shape molecule (a molecular weight of 15.5 kDa) with a dimension of approximately 24 nm-width and 10 nm-height (Fig. 5). In spite of the large size, the antigen binding site (i.e., paratope) of the antibody is extremely small entity consisting of only 3 to 5 amino acids out of total 1,320 and also localized at the end of each arm in the F_{ab} region. These features unique to an antibody may create a distinct aspect in its immobilization on a solid matrix.

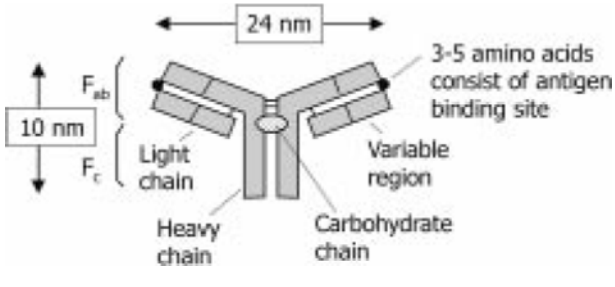

Fig. 5. A schematic presentation of the antibody molecule in a class of immunoglobulin G.

Upon addition to the solid support, the antibody molecule during an initial time period may weakly interact with the support and provide a condition for a lateral diffusion of this molecule along the surface. The diffusion process could continue until the most thermodynamically stable state as to the interaction between the protein and the solid support reaches. The next molecule colliding with the surface could undergo the identical procedure of mass transfer and, in this circumstance, it may additionally interact with the immunoglobulin already immobilized. This results in the antibody molecules present in proximity to one another. A repetition of the same process eventually creates a cluster or patch of protein molecules on the solid surface.

The complex density of an antigen with the immobilized antibody increases proportionally to the surface concentration of the antibody, but it soon reaches a maximum limit depending primarily on the antigen size (Fig. 6, left) [Cantarero et al., 1980; Schramm and Paek, 1992]. The maximal complex density is higher for the smaller antigen (^{125}I-P, MW 730) than for the larger antigen conjugated to an enzyme (P-HRP, MW 40,000). At these antibody concentrations, however, maximal packing of immunoglobulin on the surfaces has not been attained. If the antibody molecules are randomly distributed on the surface, there should be sufficient space for the large antigen to allow lateral access to the binding sites. These experimental results support that, as mentioned above, the antibody molecules occupy the surface in clusters or patches. The sizes of these patches increase when incubated with an increasing concentration of antibody although the number of binding sites accessible to the antigen is almost constant (Fig. 6, right). If the molecular size is extremely large as for plasma lipoprotein particles, the maximum complex density attainable is relatively low and, therefore, a complete immuno-separation of a single lipoprotein with an immobilized antibody may become difficult.

3. Immune Reactions in a Liquid Phase

Since the yield of complex formation between the lipoprotein and antibody at liquid-solid interface was low, a method for carrying out the reactions in a liquid phase was investigated. To attain this

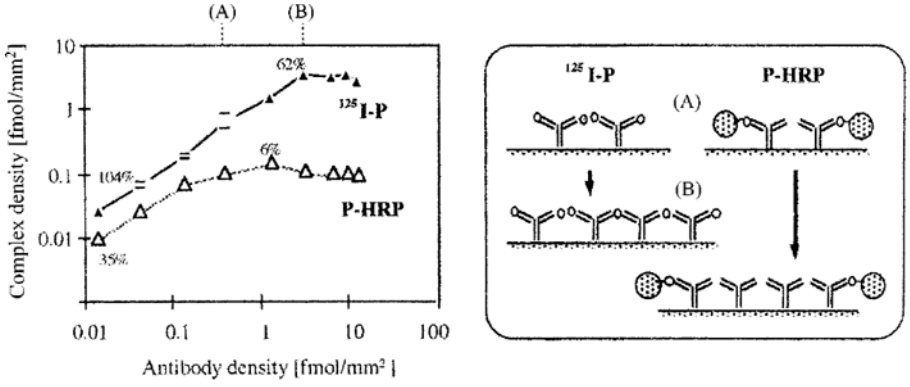

Fig. 6. Binding complex formation of different antigens in the size with an immobilized antibody (left) and a schematic presentation describing dissimilar accessibilities of small and large antigens to the antibody (right).

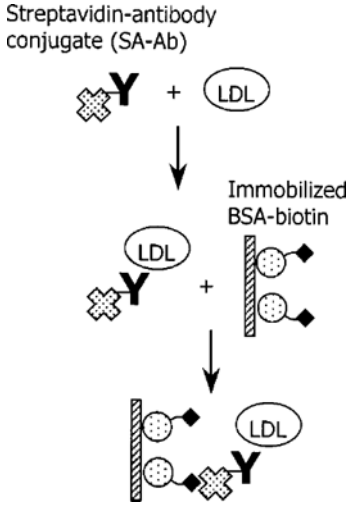

Fig. 7. Immuno-separation of lipoprotein particles based on streptavidin-biotin capture method.

goal, we designed a capture system utilizing streptavidin-biotin binding with the highest binding constant (order of 10^{15} L/mol) among known biological reactions [Weber et al., 1989]. Biotin chemically linked to BSA as carrier replaced immunoglobulin on the antibody pad (now, converted to 'biotin pad') and streptavidin was conjugated with the antibody (SA-Ab) specific to apo B100. After the conjugate, SA-Ab, reacted with LDL to form an antigen-antibody complex in a liquid phase, this mixture was absorbed from the bottom end of the biotin pad (Fig. 7). The streptavidin-biotin reaction took place on the biotin pad, and an effective removal of the lipoprotein was indeed achieved (SA-Biotin in the right of Fig. 4).

Such effectiveness in the elimination of LDL particles is attributed mainly to a free accessibility of the lipoprotein particles to the antigen binding sites of antibody since the immuno-reaction was carried out in a liquid phase. The multiple binding sites present on a streptavidin molecule, conjugated to an immunoglobulin, may also improve the probability of the capture on the biotin pad if compared to a monovalent binding [Paek et al., 1999]. This condition would improve the reaction yield especially under non-equilibrium conditions of immuno-chromatography described in this communication. The capture efficiency further increases by adapting a long, flexible linkage between biotin molecules and BSA in the conjugate.

The immuno-separation of HDL by using streptavidin-biotin capture system was specific and efficient in clinical concentration ranges of plasma lipoproteins [Paek et al., 1999]. Binding tests for each lipoprotein revealed that apo B100-containing lipoproteins, LDL and VLDL, were bound on the biotin pad while HDL did not participate in the binding reaction. However, the fractional capture of lipoproteins, estimated by the reduction in the signal as demonstrated in Fig. 4, decreased as the lipoprotein concentration increased. The capture rate was equal to or higher than 90% for LDL-C between 100 and 200 mg/dL, and 92% for VLDL-C between 20 and 40 mg/dL. The immuno-chromatographic procedure was simple and fast compared to conventional methods [Mackness and Durrington, 1992; Skinner, 1992] and, therefore, can eventually be used for point-of-care testing.

4. Dose Responses

The analytical system developed for HDL-C as described above was assessed as to selectivity by utilizing samples of lipoprotein mixtures as previously reported [Paek et al., 1999]. These mixtures as standard samples contained LDL and VLDL, adjusted to typical clinical concentrations of cholesterol (150 mg/dL LDL-C and 20 mg/dL VLDL), in the combination of HDL at different concentrations (0 to 100 mg/dL). This setting allowed us to obtain dose responses of the system to the analyte. Without using antibody to apo B100, the system produced high signals as responses to variable doses. In the proposed conditions with the antibody, the system did not only reduce significantly the signals, but also showed a pattern of dose response in proportion to the levels of HDL-C. Such dose-response pattern was approximately identical to that obtained with samples in which HDL was solely present. These results indicated that the membrane strip system can be utilized to quantitatively detect HDL-C provided a sensor, converting the cholesterol concentration to a physically measurable signal, is incorporated into the analytical system.

5. Electrochemical Immunosensor

Hydrogen peroxide, a product from enzymatic reactions of cholesterol (refer to Fig. 1), is an electrochemically active substance that can be decomposed to produce electrons upon application of a voltage. These are then transferred into an electrode surface and induce a current increase in proportion to the cholesterol concentration. To

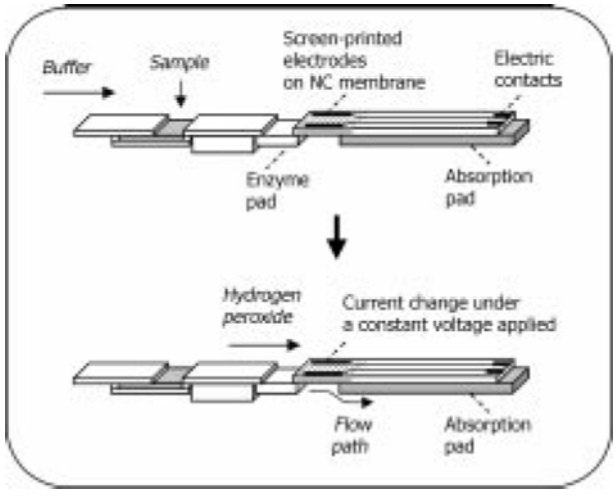

Fig. 8. Membrane strip immunosensor for a single-class plasma lipoprotein cholesterol employing an electrochemical detection technology.

realize such a quantitative method based on amperometry, we fabricate carbon/silver electrodes directly screen-printed on NC membrane as solid matrix, and replace the signal pad of the analytical system with the new membrane (Fig. 8). The electrodes are patterned in a planar structure consisting of working, counter, and reference electrodes. Electric contacts extending from the sensor part are printed on a plastic film acting as an insulator against the medium flow. The aqueous solution absorbed from the bottom of the strip system will transfer hydrogen peroxide to the membrane with electrodes and then directly flow into the absorption pad. As an alternative approach, the colorimetric signal shown in Fig. 1 can also be converted to optical density by using, for instance, a scanning photometry and charge-coupled device.

6. Bio-Micro System

The membrane strip system as diagnostic device requires, as in most clinical tests, a sample of approximately 10 to 100 μL drawn from a peripheral blood by pricking the skin. Such invasive sampling is not preferred and, thus, a method alleviating pain is needed to investigate in the near future. Although a significant reduction in the volume of sample down to a nanoliter scale may satisfy the need of non-invasive diagnosis, such extremely small volume cannot be handled in the membrane strip with a macro dimension. To attain the goal, we are devising a miniaturized system by employing a micro-electro-mechanical system (MEMS) technology. This micro system combines with biological components and bio-reactions that are required for the measurement of a single-class plasma lipoprotein cholesterol, and also adopts an integrated circuit for the signal amplification, analog-to-digital conversion, and registration.

In conclusion, we have developed an analytical concept that can be utilized to construct a point-of-care device measuring a single-class plasma lipoprotein cholesterol such as HDL-C and LDL-C. The next step of this research for constructing a user-friendly device is the automation of two discrete processes for immuno-separation, i.e., antigen-antibody reactions in a liquid phase and the subsequent fractionation based on SA-biotin binding, such that users merely add a specimen to the device without handling reagents. This can be accomplished by precisely programming the times for the reactions and separation via a control technology of liquid medium flow. Furthermore, a non-invasive micro system manipulating a nano liter-size sample as mentioned is being investigated in parallel with the automation.

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ERRATA

Lee, D.-H., Epstein, N. and Grace, J. R., "Hydrodynamic Transition for Fixed to Fully Fluidized Beds for Three-Phase Inverse Fluidization," *Korean J. Chem. Eng.*, **17**, 684 (2000).

1. Page 684, column 2, lines 5-6: Delete the word "inverse".
2. Page 686, column 1, lines 7-8 under RESULTS AND DISCUSSION: "In the case of the gas holdup correlation of Buffiere and Moletta [1999] for bubble column" should be changed to "In the case of the two gas holdup correlations of Buffiere and Moletta [1999] for bubble columns".
3. Page 688, column 2, Fig. 8: "Ibrahim et al. [1997] should be changed twice to "Ibrahim [1997]", i.e. delete "et al.".
4. Page 687, Eq. (14): $\Delta p_{particle} = \frac{M_p g\left(\frac{\rho_l}{\rho_p} - 1\right)}{A_t}$ should be changed to $\Delta p_{particle} = \frac{M_p g\left(\frac{\rho_l}{\rho_s} - 1\right)}{A_t}$.