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Improvement of lipoprotein separation with a guard channel prior to asymmetrical flow field-flow fractionation using fluorescence detection

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ABSTRACT

In this article, a simple experimental approach to improve lipoprotein separation and detection in flow field-flow fractionation (FlFFF) is detailed. Lipoproteins are globular particles composed of lipids and proteins in blood serum and their roles include transferring fats and cholesterols through blood vessels throughout the body. Especially, presence of small, dense low-density lipoproteins (LDL) is associated with cardiovascular risk. Two experimental approaches were explored in this study: an increase in the reproducibility of LDL particle separation by implementing a guard channel prior to an asymmetrical FlFFF (AFlFFF) channel in order to deplete small molecular weight serum proteins and reducing the required injection volume of a serum sample by implementing fluorescence detection. The guard channel was made of a simple hollow fiber module so that the serum sample can be washed with the help of radial flow prior to injection into the AFlFFF channel. The channel was tested with protein standards and serum samples to ensure precision of the retention time and the protein recovery rate. A fluorescent phospholipid dye was utilized to label lipoprotein particles before separation for fluorescence detection, which resulted in a reduction of the required injection volume of serum.
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1. Introduction

Lipoproteins are globular complexes in blood containing lipids and proteins, and the major role of lipoproteins is to transport cholesterols and fats throughout the body [1]. Serum lipoproteins are typically classified into high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL). Clinical analyses of the lipoprotein profile are commonly utilized for the diagnosis or risk assessment of atherosclerosis. In particular, small dense LDL is associated with an increased risk of developing cardiovascular disease [2–4]. Characterization of LDL particles is performed using several analytical methods including a density gradient ultracentrifuge [5] for density profiling, polyacrylamide gel electrophoresis (PAGE) [2,6,7], and size exclusion chromatography (SEC) [8,9] for size analysis. While ultracentrifuge and PAGE methods can yield accurate density profiles and size information for lipoprotein particles, respectively, they require a considerable amount of time and thus, are difficult to apply to large numbers of samples. SEC works well when suitable standards are provided. However, the possibility of sample interaction with the pores of packing materials always exists.

Flow field-flow fractionation (FlFFF) is a technique capable of separating nanoparticles or proteins based on hydrodynamic size differences in an open channel by the simultaneous movement of a migration flow and a crossflow, where the crossflow is applied perpendicular to the migration flow [10–12]. Crossflow drives sample components toward one wall of the FlFFF channel, and this driving force is counter-balanced by the diffusive forces of sample materials lifted from the wall at a certain position. The equilibrium height is determined by the hydrodynamic size of a sample component and the field strength or crossflow rate. Since the linear velocity of the migration flow increases as the distance from the channel wall increases, smaller particles at higher equilibrium locations migrate earlier than larger particles. Since FlFFF separation is carried out in an unobstructed channel space, it is suitable for handling macromolecules or particles without incurring interactions with packing media that often occur in chromatography or gel-based methods.

The capability of FlFFF for the size fractionation of lipoproteins has previously been demonstrated [13]. It has also been utilized for the size determination of HDL and LDL with VIS detection using Sudan Black B for selective detection of lipoproteins from blood serum samples of CAD patients [14]. A miniaturized AFlFFF channel [15] was employed to study aggregation and fusion of lipoproteins, and a multiangle light scattering (MALS) detector [16] can be utilized for the size comparison (hydrodynamic

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diameter vs. radius of gyration) of lipoprotein particles from a healthy blood sample. Recently, asymmetrical FlFFF was utilized for the enzymatic determination of cholesterol and triglycerides in serum lipoprotein by implementing on-line dual detection methods [17,18]. Hollow fiber FlFFF (or HF5), an alternative channel system using a polymeric membrane fiber as the FlFFF channel, was applied to lipoproteins as a disposable system [19]. HF5 systems have also been applied for the semi-preparative scale separation of HDL and LDL particles by assembling a multiplexed HF5 channel system [20], where the collected HDL and LDL particles were further analyzed for the profiling of phospholipids by nanoflow liquid chromatography–electrospray ionization–tandem mass spectrometry [21]. When dealing with a serum sample in FlFFF, injection of a few μL of serum introduces a considerable amount of proteins (~70 μg/μL [22]), which causes channel contamination after a few runs.

In this study, two approaches were employed to improve the separation of lipoproteins in FlFFF. A guard channel comprising a short length hollow fiber module was introduced prior to the FlFFF channel so that smaller molecular mass (<30 kDa) serum proteins would be depleted before they are introduced to the asymmetrical FlFFF (AFlFFF) channel. For the improved detection of lipoproteins with a minimized injection amount of a serum sample, a fluorescent phospholipid dye was adopted for the fluorescence detection of lipoproteins. Experiments were conducted to evaluate the performance of the guard channel in serum protein separation in terms of protein recovery and reproducibility of the retention time. Fluorescence detection was compared with VIS detection of Sudan Black B bound LDL. This combined method was applied to serum samples from CAD patients and healthy adults for the determination of the size reduction of LDL particles.

2. Experimental

2.1. Materials and reagents

The protein standards used, carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), apoferritin (444 kDa), and thyroglobulin (670 kDa), were obtained from Sigma (St. Louis, MO, USA). The blood serum samples for the lipoprotein analysis were obtained from three healthy volunteers and five CAD patients with written consensus at the Yonsei University School of Medicine. CAD patients were diagnosed by coronary angiography and healthy volunteers were students without a history of hyperlipidemia. The labeling reagents used to stain lipoproteins in the serum samples were Sudan Black B (SBB) for VIS detection and a fluorescent phospholipid dye, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (NBD-PE), for fluorescence detection. Staining of the lipoproteins in the serum samples with SBB was achieved by mixing 50 μL of raw serum with 3 μL of 1% SBB in dimethylsulfoxide. Then, the mixture was gently stirred for 20 min. For the fluorescence labeling of lipoproteins in serum, 10 μL of raw serum was mixed with 5 μL of the NBD-PE solution (3.0 μM in (1:9) dimethylsulfoxide:ethyleneglycol) and the mixture was stirred gently for 30 min at room temperature. For both staining processes, serum samples were stained every day prior to AFlFFF analysis since in some cases precipitation was observed after 2–3 days. The carrier solutions for AFlFFF were a 0.1 M PBS (phosphate buffered saline) solution adjusted at pH 7.4 for standard proteins and a 10 mM NH_4HCO_3 solution (pH 8.2) for serum samples. These solutions were prepared with ultrapure water (>18 MΩ) which was filtered before use by a 0.22 μm membrane filter (Millipore Corp., Bedford, MA, USA). It was found that stained serum sample showed reproducible results in 10 mM NH_4HCO_3 solution (pH 8.2) by AFlFFF analysis since NBD-PE needs to avoid the acidic condition. The hollow fiber membrane utilized for the preparation of the guard channel was constructed of polyacrylonitrile (PAN) with dimensions of (1.0 mm × 1.4 mm × 12 cm: i.d. × o.d. × length, 30 kDa MWCO) obtained from Chemicore Inc. (Daejeon, Korea). The channel membrane utilized for AFlFFF was regenerated cellulose with a MWCO of 10 kDa obtained from Wyatt GmbH (Dernbach, Germany).

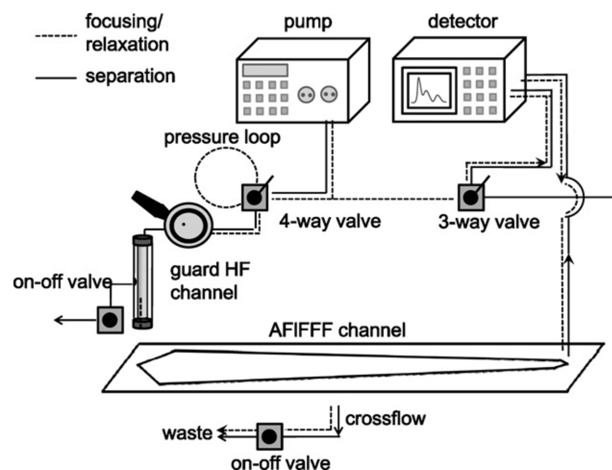

Fig. 1. Schematic illustration of the AFlFFF channel system with the guard channel washing device.

2.2. Guard HF channel and AFlFFF

The guard channel was prepared using the same method used to assemble the hollow fiber FlFFF module [20,21] and all other parameters also remained the same except for the length. A 12 cm long polyacrylonitrile (PAN) hollow fiber was inserted into a glass tube (1.8 mm i.d. and 3.5 mm o.d.), which had one end connected to a Tefzel tee supplied by Upchurch Scientific (Oak Harbor, WA, USA) for the radial flow out and the other end was connected to a union. Both ends of the guard channel module were located between a sample loop injector and the AFlFFF channel using Teflon tubes (1.5 mm o.d. and 0.0254 mm i.d.). The AFlFFF channel used was the Eclipse® 3 Channel model obtained from Wyatt GmbH (Dernbach, Germany) and was 26.6 cm in length, with a thickness of 250 μm, and a breadth that decreased from 2 cm at the beginning of the channel to the end of the trapezoidal design. Two types of sample loop injectors were utilized: a model 7125 injector (20 μL loop) for typical protein separation and a model 7525 (0.2 μL loop) injector for lipoprotein analysis with fluorescence detection, both of which were obtained from Rheodyne (Cotati, CA, USA).

The guard channel and the AFlFFF channel were connected as shown in Fig. 1. Each serum sample loaded into the injector was introduced into the guard HF channel by opening the radial flow direction only when all other outlets (crossflow and outflow exits of the AFlFFF channel) were closed. Therefore, all flows were directed to the radial flow of the guard channel and some smaller (<30 kDa) serum proteins along with the salts contained in the serum were washed off. The period of purification in the guard channel was 10 min at a radial flow rate of 0.5 mL/min. After purification, the remaining components in the guard HF channel were transferred to the AFlFFF channel when the radial flow on-off valve was off. When samples were delivered to the AFlFFF channel, they were focused at a position 1/10 of the way down the AFlFFF channel by splitting the pump flow to both ends of the AFlFFF channel using 4-way and 3-way valves, as shown in Fig. 1, so that the focusing/relaxation

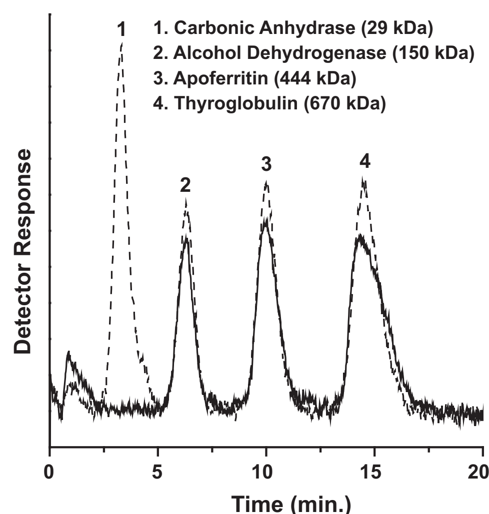

Fig. 2. AFlFFF separation of four protein standards with (solid line) and without (dotted line) using the guard channel prior to sample injection. The run conditions included a total flow rate/outflow rate ($\dot{V}_{in}/\dot{V}_{out}$) = 4.50/0.17 mL/min, guard channel washing with a radial flow of 0.5 mL/min for 10 min, and focusing/relaxation for 5 min.

process could be conducted. The focusing/relaxation period was 5 min at a total flow rate of 3.0 mL/min. After focusing/relaxation, the two valves were switched to deliver flow only to the channel inlet and the separation began. For the delivery of carrier liquid, a Model SP930D HPLC pump from Young-Lin Instrument (Seoul, Korea) was used and the pump flow was divided into two parts (1:9 or desired flow rate ratio) using a piece of capillary tubing (50 μm i.d.) by adjusting its length at the pressure loop region shown in Fig. 1. For the detection of proteins during the evaluation of the guard channel, a model UV730D UV detector (Young-Lin) was used at a wavelength of 280 nm. For the detection of lipoproteins with Sudan Black B, the same UV detector was used at 600 nm. For lipoproteins with NBD, a model RF-10A XL fluorescence detector supplied by Shimadzu (Kyoto, Japan) was utilized at an absorption wavelength of 488 nm and an emission wavelength of 520 nm. The detector signals were recorded using Autochro-Win 2.0 plus software from Young-Lin.

3. Results and discussion

The performance of a guard channel fabricated of a short HF module was tested by measuring the efficiency of protein filtration using standard proteins in the AFlFFF channel. Fig. 2 shows the superimposed fractograms of the baseline separation of carbonic anhydrase, alcohol dehydrogenase, apoferritin, and thyroglobulin obtained at an incoming flow rate/outflow rate ($\dot{V}_{in}/\dot{V}_{out}$) of 4.50/0.17 mL/min. The dotted line fractogram was obtained from the protein mixtures without the washing step at the guard channel and the solid line represents data that were obtained after washing at 0.5 mL/min for 10 min. The injected amount was 1 μg for each standard. A focusing/relaxation period of 5 min was applied with a total flow rate of 4.50 mL/min. The recovery of each protein was calculated by comparing the peak area of each eluted protein with or without guard channel washing. For the case of carbonic anhydrase (29 kDa), its recovery after washing at the guard channel was nearly 0, as seen in the dotted line fractogram. However, the recovery percentages of the other three proteins of alcohol dehydrogenase, apoferritin, and thyroglobulin (larger than 30 kDa) were 91.3 ± 8.2%, 89.6 ± 6.8%, and 88.6 ± 6.5%, respectively. This is a satisfactory result for the depletion of smaller MW proteins, but there was some loss for the larger proteins.

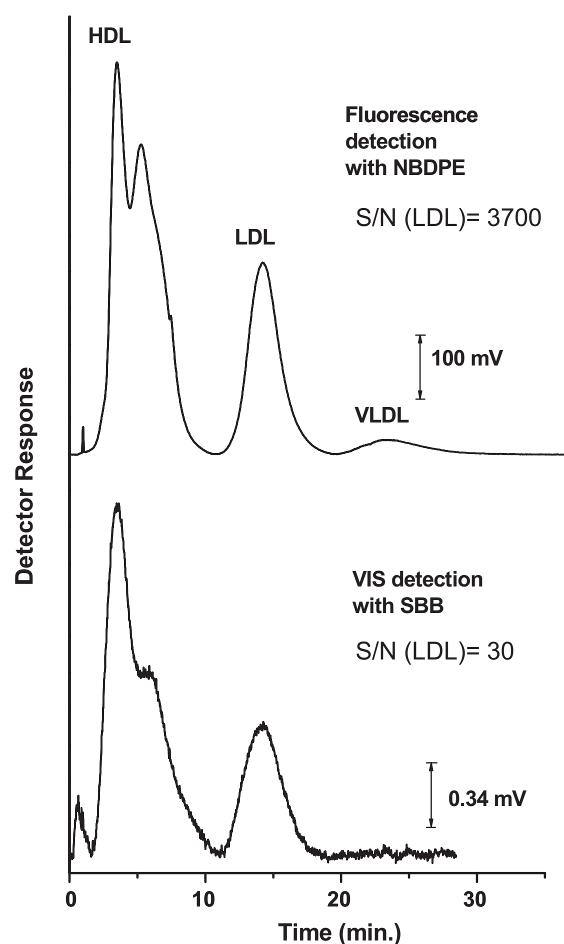

Fig. 3. AFlFFF separation of lipoproteins from a healthy human serum sample using fluorescence detection (top fractogram, NBDPE dye) and VIS detection (bottom, SBB dye) obtained with a $\dot{V}_{in}/\dot{V}_{out}$ of 3.00/0.24 mL/min.

For the separation and selective detection of lipoproteins, SBB was utilized to stain serum lipoproteins with VIS detection in our previous studies [14,21]. In the present work, the injected amount of a stained serum sample was minimized to 2 μL (1.9 μL pure serum and 0.1 μL dye). In order to reduce the injection amount, which also reduces the chance of channel contamination, NBDPE, a fluorescence dye, was utilized for staining lipoproteins. For the AFlFFF separation of serum lipoproteins, the run conditions applied in Fig. 2 were not appropriate since LDL particles are much larger than typical blood proteins. Thus, a relatively fast run condition, $\dot{V}_{in}/\dot{V}_{out}$ of 3.00/0.24 mL/min, was selected for the separation results in Fig. 3. The top fractogram shown in Fig. 3 represents the fluorescence detection (FD) signals (λ_{abs} = 488 nm and λ_{em} = 520 nm) of a serum sample using NBDPE with an injection volume of only 0.2 μL (0.13 μL portion of pure serum and 0.07 μL of dye), while the bottom fractogram represents the VIS signals (λ_{abs} = 600 nm) using SBB dye with an injection of 2.0 μL of the same serum sample (1.9 μL portion of pure serum and 0.1 μL of SBB dye). Both cases utilized the guard channel for on-line washing of the serum sample prior to injection to the AFlFFF. The intensity of the FD signals was approximately 120 times (3700 vs. 30 for the signal to noise ratio of LDL peaks *n* Fig. 3) higher than that with the SBB dye. For selective detection of lipoprotein particles, light scattering detection can be utilized in conjunction with FlFFF but requires a considerable injection volume of serum (10 μL for the commercial AFlFFF channel and 4 μL for the HF5 channel [16]). The methods used in the current study not only reduced the

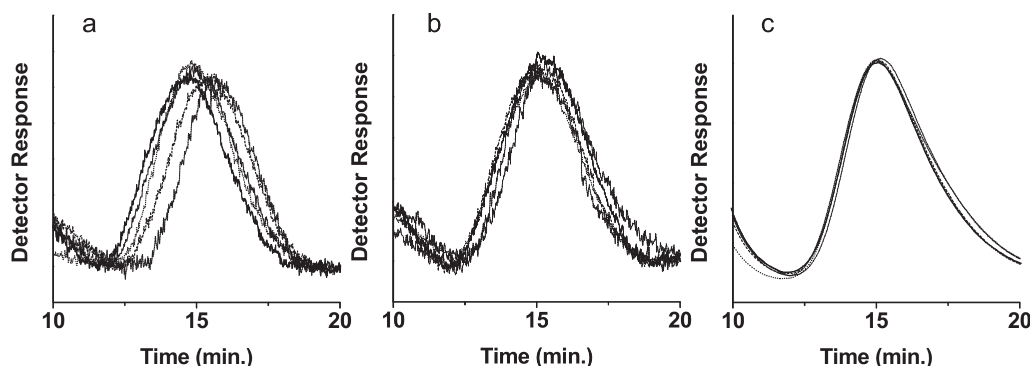

Fig. 4. Comparison of enlarged fractograms of LDL with repeated measurements by AFlFFF: (a) VIS detection using SBB without the guard channel (1.9 μL serum and a total injection of 2.0 μL), (b) VIS detection using SBB with the guard channel, and (c) fluorescence detection using NBDPE with the guard channel (0.13 μL serum and a total injection of 0.20 μL) where $\dot{V}_{in}/\dot{V}_{out} = 3.00/0.22$ mL/min.

required injection volume of the serum sample but also allowed the selective detection of lipoproteins in the serum sample without detecting serum proteins of similar size to lipoproteins encountered when light scattering detection is utilized. Moreover, a small trace of VLDL is also seen in Fig. 3 when FLD with NBDPE was utilized.

The effect of the guard channel on the reproducible elution of LDL particles was tested and the fractograms resulting from repeated runs are shown in Fig. 4. In Fig. 4, the fractograms of five repeated runs are displayed (shown with peaks after 10 min for comparison) for LDL particles from a healthy human serum sample before and after applying the guard channel washing procedure. In Fig. 4a and b, SBB was stained before injection, and detection of LDL was performed at a wavelength of 600 nm. The retention time of LDL in Fig. 4a was 15.14 ± 0.58 min without using the guard channel, which reflects ∼4% fluctuation in the retention time. However, after using the guard channel, the fluctuation decreased to less than 1% (15.13 ± 0.12 min), as seen in Fig. 4b. As shown in Fig. 4c, the reproducibility of the retention time can be improved further with less deviation (∼0.4%, t_r = 15.08 ± 0.06 min) when NBDPE dye is utilized with the guard channel washing and with an injection of 0.2 μL of the stained serum sample. It is likely that by using the guard channel, the reproducibility of the retention time can be greatly increased. The explanation for this behavior is that some of the small MW serum proteins are depleted in the guard channel and thus, channel contamination is reduced. Even though typical abundant serum proteins have molecular masses larger than 30 kDa (albumin 66 kDa and IgG 150 kDa, which together occupy up to 80–85% of the mass of serum), small MW proteins may block the pores of the channel membrane under the crossflow movement, causing fluctuations in the crossflow uniformity and therefore, variations of the retention time.

A guard channel washing process with fluorescence detection was applied in order to distinguish the differences in the LDL sizes between healthy and CAD patients. In Fig. 5, the fractograms of three healthy serum samples and five CAD patient samples obtained by using fluorescence detection in AFlFFF are shown. The injection amounts for all runs were fixed at 0.2 μL (0.13 μL portion of pure serum) and the run conditions included 10 min of washing at the guard channel under a radial flow of 0.5 mL/min, 5 min for focusing/relaxation at AFlFFF, and a $\dot{V}_{in}/\dot{V}_{out}$ flow rate ratio of 3.00/0.11 mL/min. Fig. 5 shows that the peak heights of the LDL of each individual sample are different from each other, indicating that the amount of LDL differs between individuals. However, the average LDL retention time for patient samples was 19.8 ± 0.3 min (n = 5) (equivalent to 23.2 nm based on FlFFF theory [11]) while that of the control samples was 21.4 ± 0.4 min (n = 3) (25.1 nm). For the detailed comparison of the LDL sizes between control and patient

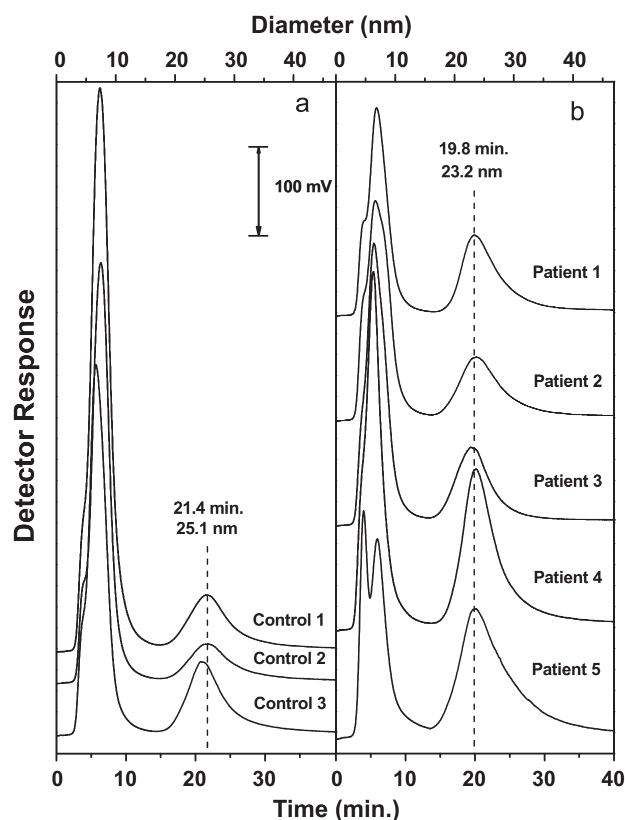

Fig. 5. Superimposed fractograms of lipoprotein for (a) three control samples and (b) five CAD patients obtained at $\dot{V}_{in}/\dot{V}_{out} = 3.00/0.11$ mL/min using guard channel washing and fluorescence detection with NBDPE. The injection volume was 0.2 μL (0.13 μL serum).

samples, a systematic comparison using more number of samples should be carried out.

4. Conclusions

The present study introduces a guard channel washing procedure to deplete small MW serum proteins prior to injection into the AFlFFF channel and adopts fluorescence detection of lipoproteins using a NBDPE dye. The use of a guard channel (hollow fiber with 30 kDa molecular weight cut off) prior to AFlFFF separation allowed smaller MW proteins to be depleted efficiently, which may reduce the contamination of the AFlFFF channel membrane and therefore, enhance the reproducibility of the retention time of LDL particles in

the serum samples. Compared to VIS detection using Sudan Black B, fluorescence detection using NBDPE along with guard channel washing can reduce the required injection amount of the serum sample to 0.13 μL, which also decreases the chance of channel contamination by adsorption of serum proteins. The results of this study demonstrate that the introduction of a guard channel to AFlFFF with fluorescence detection can be utilized to characterize LDL size for CAD patients with improved reproducibility.

Acknowledgement

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