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Fluorescence immunoassay of *E. coli* using anti-lipopolysaccharide antibodies isolated from human serum

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ABSTRACT

In this work, the gram-negative bacterium *Escherichia coli* strain BL21(DE3) (with lipopolysaccharide (LPS) in its outer membrane) and its modified ClearColi™ strain (lacking LPS) were used for the separation of anti-LPS antibodies from human serum by the following steps: (1) binding of the serum proteins to BL21(DE3); (2) dissociation of the bound proteins (including anti-LPS antibodies) from BL21(DE3) with acid; (3) filtering of the dissociated proteins using ClearColi to remove unwanted proteins; and (4) separation of the antibody fraction by protein-A column chromatography. The binding properties of the separated antibodies were analyzed by fluorescence-activated cell sorting to confirm their selective binding to LPS on the outer membrane of BL21(DE3), and by thermophoretic immunoassay to estimate their dissociation constant. The in vitro applicability of the separated anti-LPS antibodies was demonstrated through a fluorescence assay of BL21(DE3), after immobilizing the antibodies onto a modified microplate surface. The electrochemical detection of BL21(DE3) was also achieved after immobilizing the anti-LPS antibodies onto a gold electrode.

1. Introduction

The outer membrane of gram-negative bacteria such as *Escherichia coli* is covered by the endotoxin lipopolysaccharide (LPS). As shown in Fig. 1(a), LPS is composed of lipid A covalently bonded to a polysaccharide molecule made up of inner and outer cores and an O-antigen. This endotoxin has been shown to be a strong pyrogen that causes a typical fever in animals when administered by intravenous or intraperitoneal injection (An et al., 2018; Porat et al., 1995; Ravanelli et al., 2007; Steiner et al., 2006). The mechanism by which LPS promotes the proliferation of B cells was reported to be related to immunoglobulin (IgG) production (Klasen et al., 2018; Zhang et al., 2011). Recently, the LPS-free *E. coli*, named ClearColi™, was reported to modify the synthesis of LPS through the incorporation of seven gene deletions (Δ*gutQ*, Δ*kdsD*, Δ*lpxL*, Δ*lpxM*, Δ*pagP*, Δ*lpxP*, and Δ*eptA*; shown in Fig. 1(b)), which removed all the carbohydrate chains that are usually attached to the LPS molecule (Kilár et al., 2013; Mamat et al., 2015; Raetz and Whitfield, 2002; Ueda et al., 2016; Walsh and Wencewicz, 2014).

As animals and humans have been infected by LPS-carrying *E. coli* strains, human serum is expected to include anti-LPS antibodies, and many efforts have been made to detect and purify such antibodies from human serum (Chan et al., 2014; Jäkel et al., 2008; O'Shaughnessy et al., 2013; Patris et al., 2016). For example, antibodies against the O-polysaccharide of *Salmonella Typhimurium* were purified from healthy adults in order to make neutralizing antibodies for children and HIV-infected adults suffering from bacteremia in Sub-Saharan Africa (O'Shaughnessy et al., 2013). As another example, sera from healthy infants (under 1 year old), toddlers (3–4 years), and adults (18–65 years) were assayed for their ability to bind to inner core LPS epitopes of *Neisseria meningitides,* and naturally occurring serum human antibodies to *N. meningitidis* LPS were reported to access the inner core epitopes of encapsulated organisms with fully extended LPSs (Jäkel et al., 2008). Most of the reported works to separate antibodies against LPS had used affinity chromatography to immobilize the purified O-polysaccharides or the whole LPS molecule onto chromatography resins.

In this work, the *E. coli* strain BL21(DE3) (with LPS in its outer membrane) and its modified ClearColi (lacking LPS) were used to separate anti-LPS antibodies from human serum. As shown in Fig. 1(c),

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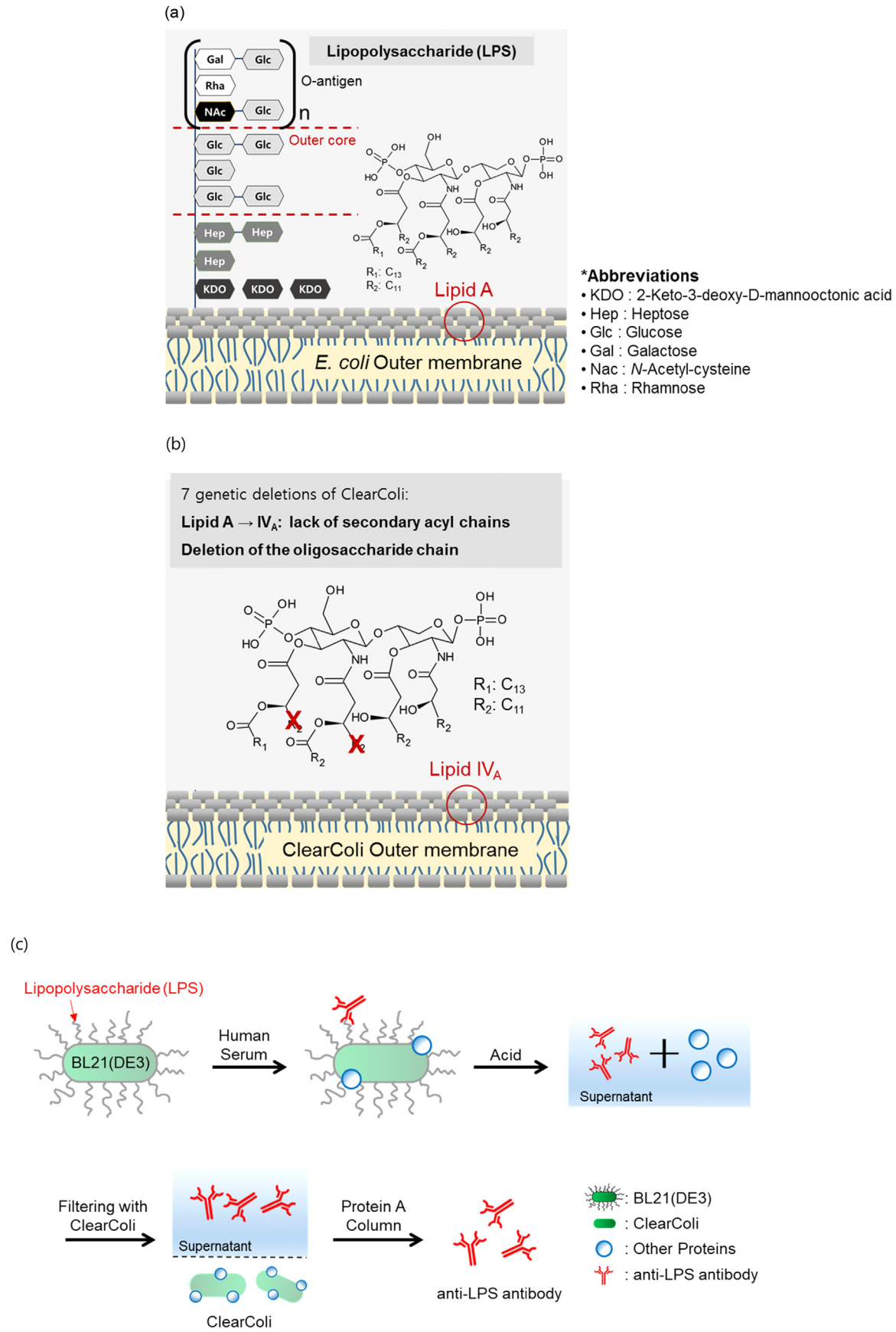

Fig. 1. Structure of the outer membrane of gram-negative bacteria. (a) Structure of the lipopolysaccharide (LPS) on the outer membrane of gram-negative bacteria. (b) Structure of the outer membrane of ClearColi. (c) Steps for separating the anti-LPS antibodies from human serum.

the separation process involved the following steps: (1) binding of the serum proteins to BL21(DE3); (2) dissociation of the bound proteins (including anti-LPS antibodies) from BL21(DE3) with acid; (3) Filtering of the dissociated proteins using ClearColi; and finally (4) separation of the antibody fraction by protein-A column chromatography. In the first step, the binding property of the separated antibodies was analyzed by (1) fluorescence-activated cell sorting (FACS) to confirm their selective binding to LPS-carrying bacteria, and (2) thermophoretic immunoassay to estimate the dissociation constant (Hu et al., 2018; Lee et al., 2017). The applicability of the purified anti-LPS antibodies was demonstrated by analyzing their ability to detect gram-negative bacteria in vitro, after their immobilization onto either a microplate surface or a gold electrode.

2. Experimental

2.1. Materials

Human serum (H4522), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), lysozyme, DNase, Triton X-100, Tween-20, 2× YT medium, and antibiotics (carbenicillin, kanamycin, and chloramphenicol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) broth was purchased from Duchefa (Haarlem, The Netherlands). Aprotinin was acquired from Roche (Basel, Switzerland). Cy3- and fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (anti-hIgG) were purchased from Abcam Co. (Cambridge, UK).

2.2. Separation of anti-LPS antibodies

E. coli strains BL21(DE3) and ClearColi™ BL21(DE3) were used to separate anti-LPS antibodies from human serum. BL21(DE3) was a modified strain from BL21 to have the coding sequence of T7 RNA polymerase which was require for the protein expression system based on T7 promotor (Studier and Moffatt, 1986). The *E. coli* strains including ClearColi, BL21(DE3), HB101, DH5α, JM110 and *B. subtilis* were cultured at 37 °C in LB broth containing 10 μM ethylenediaminetetraacetic acid (EDTA) for 16 h. To obtain green fluorescent protein (GFP)-expressing cells, plasmid pKE019 was transformed into the *E. coli* cells by electroporation and the strain was grown in LB medium containing 15 μg/mL chloramphenicol for 16 h. The cultured *E. coli* BL21(DE3) cells (1 mL) were harvested by centrifugation at 7000*g* for 3 min and washed three times with PBS (1 mL). The cells were resuspended in PBS and then, the optical density was measured at the wavelength of 600 nm (OD_{600}) using a microplate reader (Versamax, Molecular Devices, San Jose, CA, USA), and the final concentration was adjusted to be OD_{600} = 1.0 which was corresponded to 5×10^8 cells/mL (Bong et al., 2014; Mandakhalikar et al., 2018; Raymond and Champagne, 2015; Volkmer and Heinemann, 2011).

The outer membrane fraction of BL21(DE3) including LPS was isolated by an enzymatic method. The harvested *E. coli* cells were and lysed by lysozyme (200 μg/mL in 20 mL of Tris-HCl buffer (60 mM, pH 8.0) with 20 mM sucrose and 0.2 mM EDTA) for 10 min at RT. And then PMSF (1 mM) and aprotinin (20 μg/mL) were added. The OMs of *E. coli* cells were isolated by the addition of 40 mL of extraction buffer (2% Triton X-100, 50 mM Tris-HCl, 10 mM $MgCl_2$) with DNAse (10 μg/mL). After incubation for 30 min on ice, the lysate was centrifuged at 5000 g for 5 min the supernatants (corresponding to purified cell lysates) were transferred to a new centrifugation tube and centrifuged at 35,000 g for 10 min in order to acquire the outer membrane protein fraction. The separated outer membrane fraction was dissolved in 100% fetal bovine serum containing 0.2% Tween-20 for the thermophoretic immunoassay.

The anti-LPS antibodies in the human serum were separated using the following steps. (1) The human serum (1 mL) was incubated with LPS-carrying BL21(DE3) cells (10^7 cells) for 1 h under mild mixing condition (20 rpm) by using a wheel-rotating mixer (RT-10 from Daehan Scientific Co, Wonju, Korea). (2) The bound proteins (including anti-LPS antibodies) were dissociated from BL21(DE3) by treatment with 1 mL of 0.1 M glycine-HCl buffer (pH 2.7) for 10 s. And then, the reaction was neutralized by the addition of 55 μL of 1 M Tris-HCl buffer (pH 10.5). (3) The dissociated proteins were added to 10^7 cells of ClearColi to remove other unwanted proteins. This ClearColi filtering process was repeated three times. (4) The antibody fraction was separated using a protein-A column. At first, the dissociated proteins were loaded onto the protein A column. And then the column was washed with 10 mL of PBS in order to remove unbound protein. The bound anti-LPS antibodies were eluted by loading 2 mL of 0.1 M glycine-HCl buffer (pH 2.7), and eluted antibodies were directly collected in a microcentrifugal tube containing 110 μL of 1 M Tris-HCl buffer (pH 10.5). Finally, the antibodies were concentrated by Amicon centrifugal filtration (molecular weight cutoff: 3000 Da). The yield of antibodies was defined as the amount of protein separated from unit volume (1 mL) of human serum. The amount of protein was calculated using a bicinchoninic acid (BCA) assay kit from Thermo Fisher Scientific (Waltham, MA, USA).

2.3. Assays carried out with the separated anti-LPS antibodies

Flow cytometry was conducted as previously described (Park et al., 2013; Yoo et al., 2013). Cultured cells were first resuspended in PBS and adjusted to an OD_{600} of 1. The cells were then incubated in 100 μL of anti-LPS antibody (50 μg/mL) in PBS for 1 h at room temperature. After washing once with 1% PBST and twice with PBS, the *E. coli* pellet was resuspended in 100 μL of FITC-conjugated anti-hIgG antibody in PBS (10 μg/mL) for 1 h at room temperature. After the incubation, the cells were analyzed by using FACSCalibur analyzer from Becton-Dickinson Co (Franklin Lakes, NJ, USA). For cytometry of antibody-bound bacteria the level of green fluorescence (FL1) was measured for forward scattering count (FSC) in logarithmic scales.

The outer-membrane fraction was resuspended in 100% fetal bovine serum containing 0.2% Tween-20. Anti-LPS antibodies (20 μL) serially diluted in PBS were then mixed with 20 μL of the outer-membrane fraction at the concentration of 300 μg/mL. For the thermophoretic immunoassay, the sample was controlled to have 50% fetal calf serum with 0.1% Tween-20 in PBS. Each prepared sample was loaded into capillaries with a diameter of 100 μm (Nanotemper Technologies, Munich, Germany), and the thermophoretic immunoassay was performed using the Monolith NT.115 instrument (Nanotemper Technologies). Signals were measured at 80% LED power (excitation: 625 nm; emission: 680 nm) and 60% infrared laser power at room temperature. Data analysis was carried out using the instrument-integrated MO.Affinity Analysis software.

For the fluorescence immunoassay, *E. coli* cells were immobilized onto microplates bearing a modified surface (Jose et al., 2010; Lee et al., 2012; Park et al., 2014, 2013, 2011; Pyun et al., 2017). To create the modified surface, polystyrene microplates were coated with parylene-H film using a parylene coater and parylene-H precursors from FemtoScience Co. (Seoul, Korea). Deposition of the parylene-H film was controlled to achieve a final thickness of 20 nm by adjusting the initial amount of parylene-H precursors to 50 mg (Jeon et al., 2011; Jung et al., 2014; Ko et al., 2011; Yoo et al., 2011). To immobilize the anti-LPS antibodies onto the microplate, 10 μg/mL of antibodies was incubated in each well for 16 h at 4 °C. After washing once with 1% PBST and twice with PBS, 10 mg/mL of BSA was added to each well and the plate was incubated for 1 h. To determine the specificity and affinity of the anti-LPS antibodies, GFP-expressing *E. coli* BL21(DE3) and GFP-expressing ClearColi BL21(DE3) cells were incubated separately with the antibodies. After washing once with 1% PBST and twice with PBS, the fluorescence was measured by using Victor X5 plate reader from Perkin Elmer (Waltham, MA, USA) with F485(± 7) excitation filter and F535(± 12.5) emission filters (Jose et al., 2009; Park et al., 2010).

The Impedence spectroscopy was carried out by using three

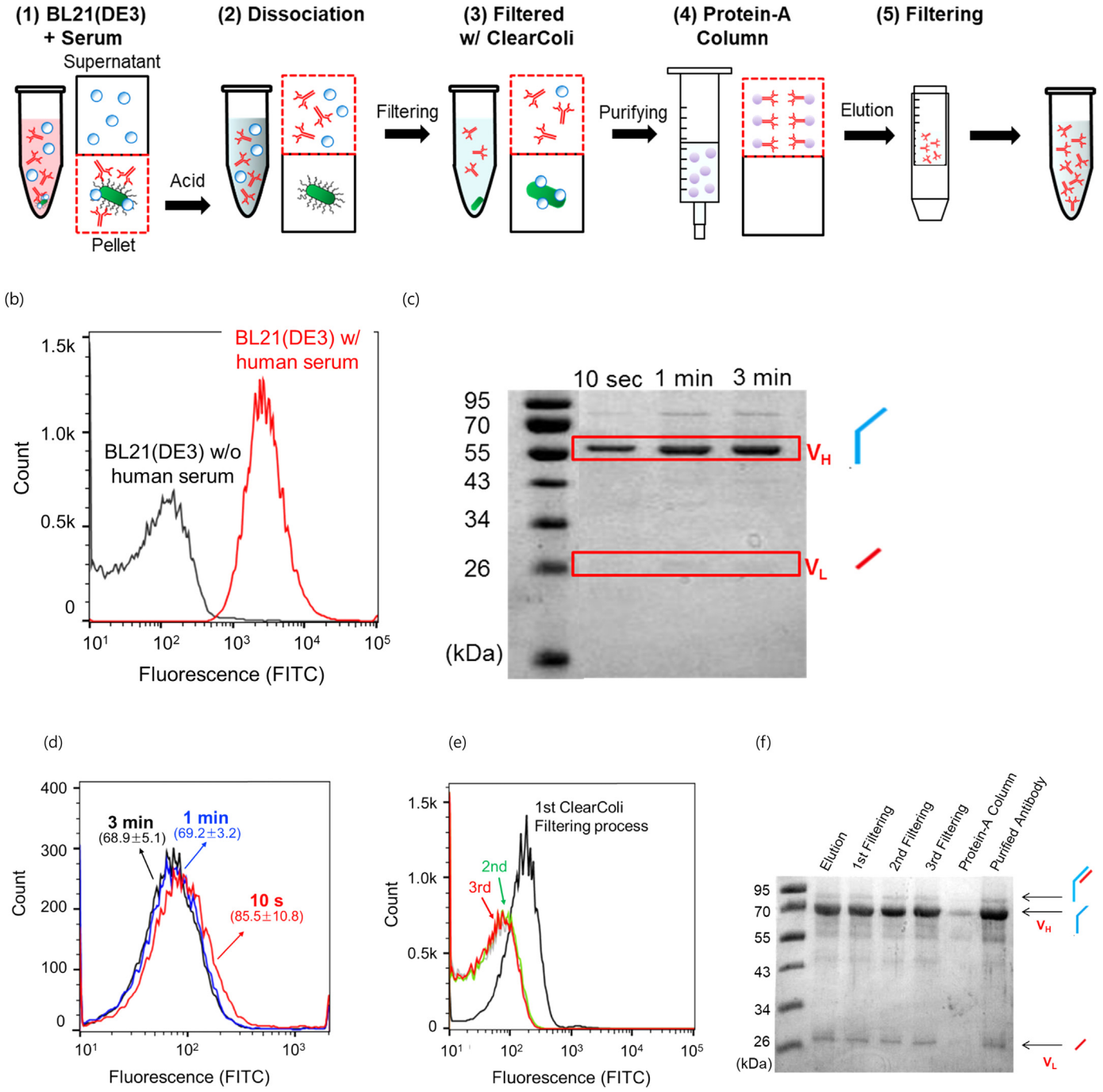

Fig. 2. Optimization of the separation of anti-LPS antibodies from serum. (a) Steps for separating the anti-LPS antibodies from human serum. (b) FACS analysis of anti-LPS antibodies bound to the outer membrane of BL21(DE3). (c) SDS-PAGE of anti-LPS antibodies dissociated from BL21(DE3). (d) Activity assay of anti-LPS antibodies dissociated from BL21(DE3), as determined by FACS. (e) FACS analysis of anti-LPS antibodies bound to the outer membrane of ClearColi after repeated filtering steps. (f) SDS-PAGE of anti-LPS antibodies in filtered and finally purified solutions.

electrode system and a commercial potentiostat from IVIUM Technologies (Eindhoven, Netherlands). As a working electrode, a gold electrode with an area of 27 mm^2 was prepared by sputtering gold layer with a thickness of 100 nm on the substrate of Si wafer. The separated anti-LPS antibodies were immobilized on the gold electrode by incubation of antibody solution at the concentration of 20 μg/mL for 2 h. The binding of bacteria was estimated after incubation of bacteria solution (100 μL) for 1 h. After washing step with 0.1% Tween 20, the impedance spectroscopy was performed in the frequency range of 0.1 Hz to 100 kHz versus the Ag/AgCl reference electrode at an amplitude of 50 mV in 50 mM potassium ferricyanide. The measured impedance was analyzed by using an equivalent circuit model, which consisted of interfacial electrode impedance modeled as the constant phase element (CPE_{el}) and a series medium resistance (R_s).

3. Results and discussion

3.1. Separation of anti-LPS antibodies from the serum

The anti-LPS antibodies in human serum were separated using the 4 steps outlined in the sections above, as shown in Fig. 2(a). The efficiency of separation of the anti-LPS antibodies was largely dependent

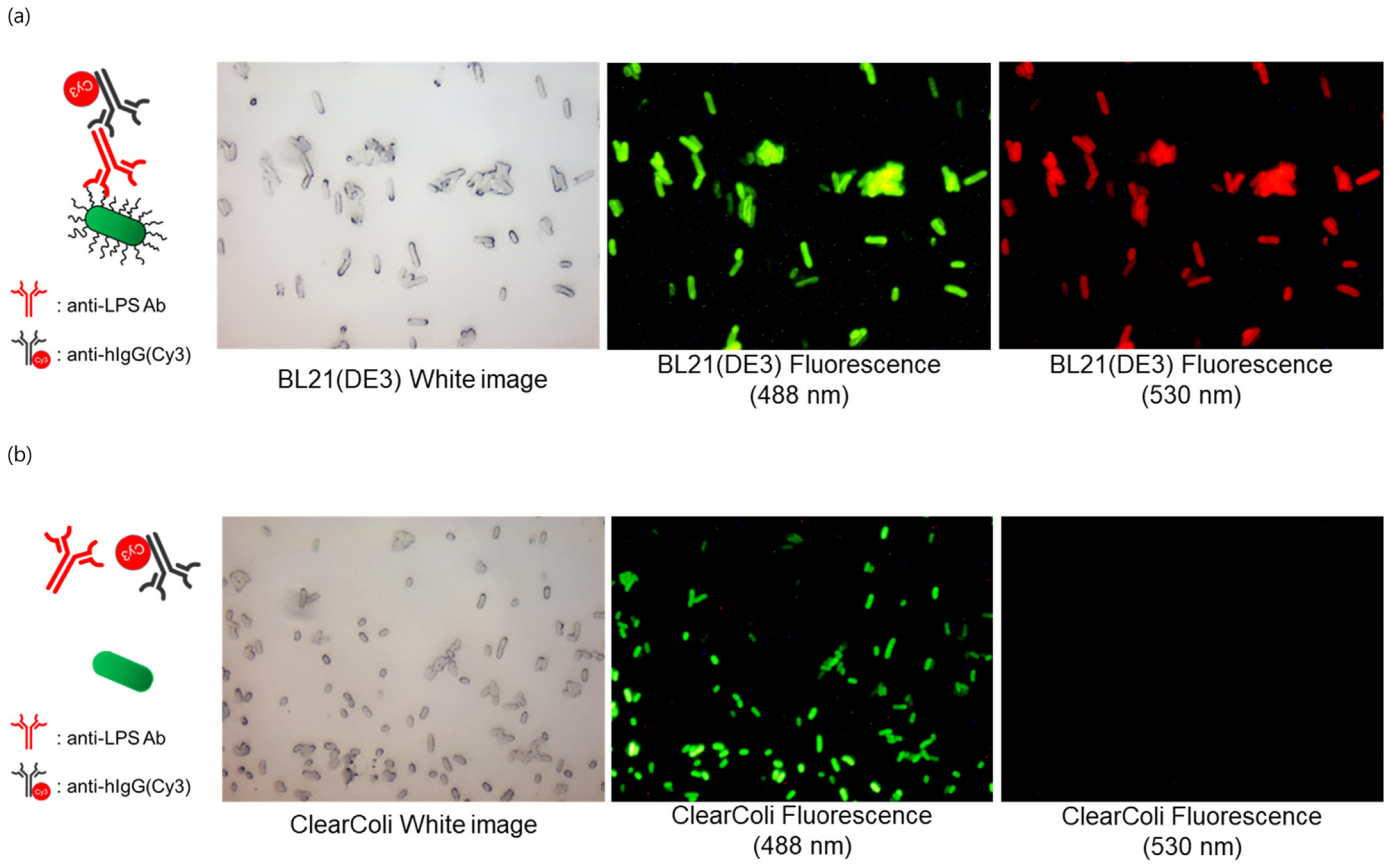

Fig. 3. Fluorescence image analysis of the binding of separated anti-LPS antibodies. (a) Fluorescence images of green fluorescent protein (GFP)-expressing BL21(DE3) after treatment with anti-LPS antibodies, observed using Cy3-labeled anti-hIgG antibodies. (b) Fluorescence images of GFP-expressing ClearColi after treatment with anti-LPS antibodies, observed using Cy3-labeled anti-hIgG antibodies.

on the first binding step to BL21(DE3), which was estimated by FACS analysis of *E. coli* BL21(DE3) cells in human serum after their reaction with FITC-labeled anti-hIgG antibodies. As shown in Fig. 2(b), FACS analysis showed that before their incubation in human serum, the BL21(DE3) cells showed no significant fluorescence. After their incubation in human serum, there was a significant level of fluorescence, which indicated the binding of anti-LPS antibodies onto the outer membrane of BL21(DE3).

The anti-LPS antibodies bound to BL21(DE3) were dissociated by treatment with an acidic solution (0.1 M glycine-HCl, pH 2.7). To optimize the acid treatment time, the antibody-bound BL21(DE3) cells were incubated with the acidic solution for 10 s, 1 min, or 3 min, and the solution was then neutralized with Tris buffer to prevent denaturation of the antibodies. As shown in Fig. 2(c), SDS-PAGE of the antibodies dissociated from BL21(DE3) revealed an increase in their amount according to the incubation time with acid. For the activity assay of these acid-dissociated antibodies, FACS analysis was carried out after their reaction with FITC-labeled anti-hIgG antibodies. After BL21(DE3) was incubated in human serum, the antibodies bound to BL21(DE3) were dissociated by the treatment of acid for 10 s, 1 min, 3 min. For the comparison of the binding activity of the separated anti-LPS antibodies according to the acid treatment time, the antibodies were reacted with BL21(DE3) and the binding to the BL21(DE3) was estimated by treatment of anti-hIgG antibodies labeled with fluorescence and FACS analysis. As shown in Fig. 2(d), the separated anti-LPS antibodies by the acid treatment time of 10 s showed higher binding activity in comparison with those of 1 min and 3 min. From these result the optimum time for the acid treatment was determined to be 10 s.

The separation of anti-LPS antibodies with a high specificity toward the LPS of BL21(DE3) was achieved using ClearColi, which is the BL21(DE3) strain genetically modified to have no LPS in its outer membrane. The proteins dissociated from BL21(DE3) in step 2 of the separation protocol were added to the ClearColi cells, whereupon those proteins with affinity to the outer-membrane components of BL21(DE3) would bind to the outer membrane of ClearColi. The centrifugation-separated supernatant was repeatedly washed with ClearColi so that anti-LPS antibodies with affinity to LPS could be selectively separated. To estimate the optimal number of filtering steps with ClearColi, the binding of antibodies to ClearColi was analyzed by FACS after reaction of the cells with FITC-labeled anti-hIgG antibodies. As shown in Fig. 2(e), after the first filtering process of dissociated proteins from BL21(DE3), ClearColi showed a high fluorescence level, which indicated the binding of antibodies to the cells. However, the fluorescence decreased to a baseline level after two filtering processes with ClearColi. From these results, the optimal number of filtering steps with ClearColi was determined to be less than three times.

The protein profile of each step of the separation protocol was analyzed by SDS-PAGE. As shown in Fig. 2(g), the dissociated proteins from BL21(DE3), the proteins of the supernatants at each filtering process with ClearColi, and the purified antibodies showed similar gel bands with the molecular weights of 75 kDa (one heavy chain + one light chain), 55 kDa (a single heavy chain), and 25 kDa (a single light chain). The yield of proteins at each separation step changed from 100% (dissociated proteins from BL21(DE3)) to 76%, 73% and 62% at the respective filtering steps. Finally, the yield of anti-LPS antibodies after protein-A column chromatography was determined to be 10.23 ± 0.53% (n = 5). The absolute amount of anti-LPS antibodies obtained from 1 mL of human serum was 23.1 ± 7.0 μg (n = 18).

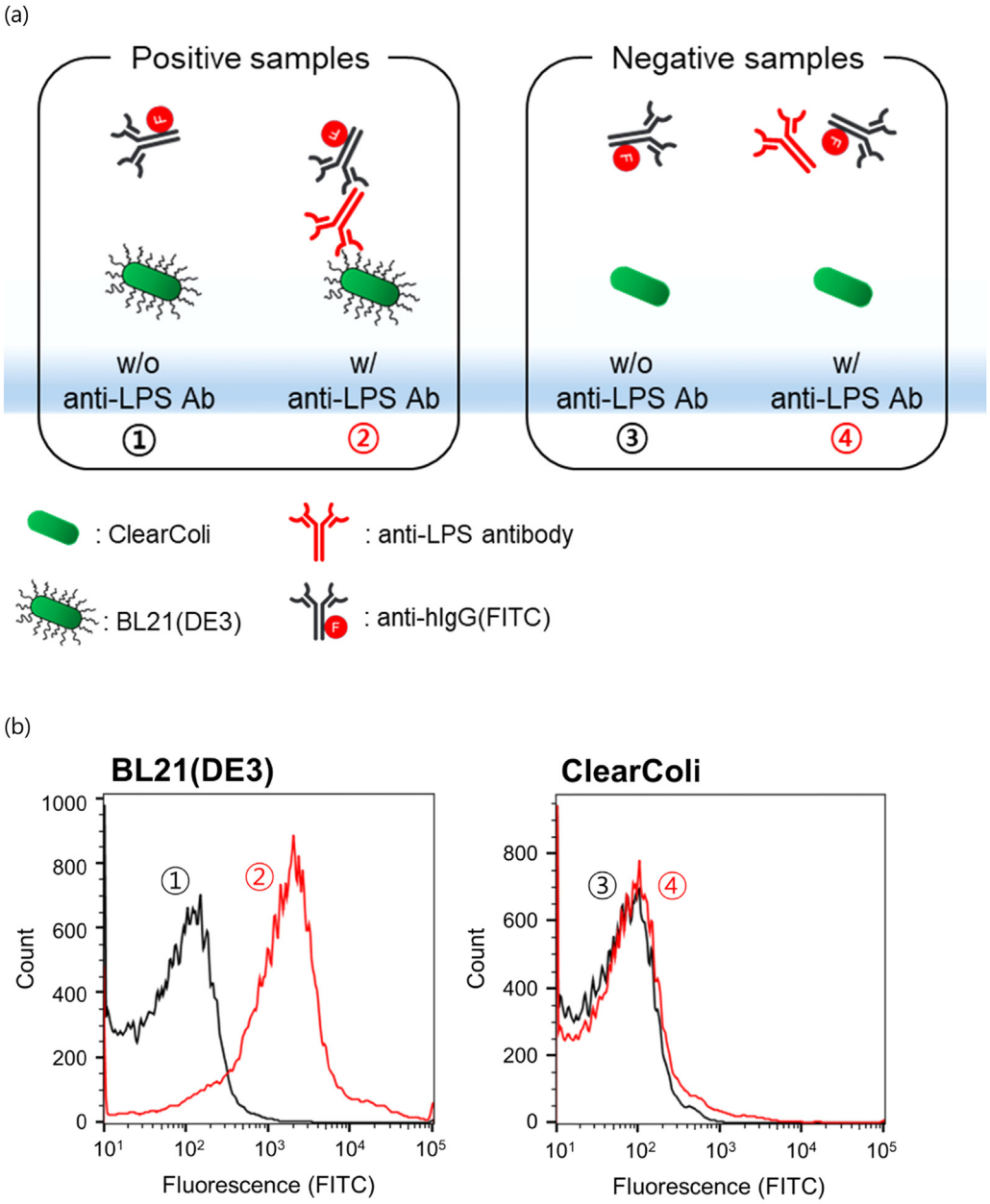

Fig. 4. FACS analysis of the binding of the separated anti-LPS antibodies. (a) Positive samples (BL21(DE3) before and after treatment with the separated anti-LPS antibodies) and negative samples (ClearColi before and after treatment with the separated anti-LPS antibodies). (b) FACS analysis with ClearColi before and after treatment with the separated anti-LPS antibodies. (c) FACS analysis with BL21(DE3) before and after treatment with the separated anti-LPS antibodies.

3.2. Characterization of the separated anti-LPS antibodies

The properties of the separated anti-LPS antibodies were first evaluated by fluorescence imaging. The separated anti-LPS antibodies were added to GFP-expressing BL21(DE3) cells, and the binding of the separated antibodies to the cells was then analyzed by using Cy3-labeled anti-hIgG antibodies. GFP-expressing ClearColi was used as the negative control. As shown in Fig. 3(a), BL21(DE3) showed green fluorescence at the emission channel of 530 ± 30 nm due to the internally expressed GFP, and red fluorescence at the emission channel of 585 ± 42 nm due to the bound Cy3-labeled anti-hIgG antibodies. These results showed that the separated anti-LPS antibodies had bound to the outer membrane of BL21(DE3). When the same binding test of the separated anti-LPS antibodies was carried out with ClearColi, green fluorescence was observed, whereas red fluorescence was not observed. These results showed that the separated anti-LPS antibodies had not bound to the outer membrane of ClearColi and that the binding of these antibodies was selective toward the outer membrane of BL21(DE3) only.

The selective binding of the separated anti-LPS antibodies in solution phase was quantitatively analyzed using FACS. BL21(DE3) with LPS (① and ② in Fig. 4(a)) and ClearColi without LPS (③ and ④ in Fig. 4(a)) were used to confirm the binding of separated antibodies. In this work, ClearColi without LPS was used as a negative sample without LPS. The separated anti-LPS antibodies were treated to both of two stains, and then anti-hIgG antibodies labeled with fluorescence were treated to monitor the binding of anti-LPS antibodies to both of two strains. In order to estimate the non-specific binding of anti-hIgG antibodies labeled with fluorescence, that is a baseline of fluorescence, was monitored by the reaction without the treatment of anti-LPS antibodies (① and ③ in Fig. 4(a)). The binding of anti-LPS antibodies to both of two strains was monitored by treatment of anti-hIgG antibodies

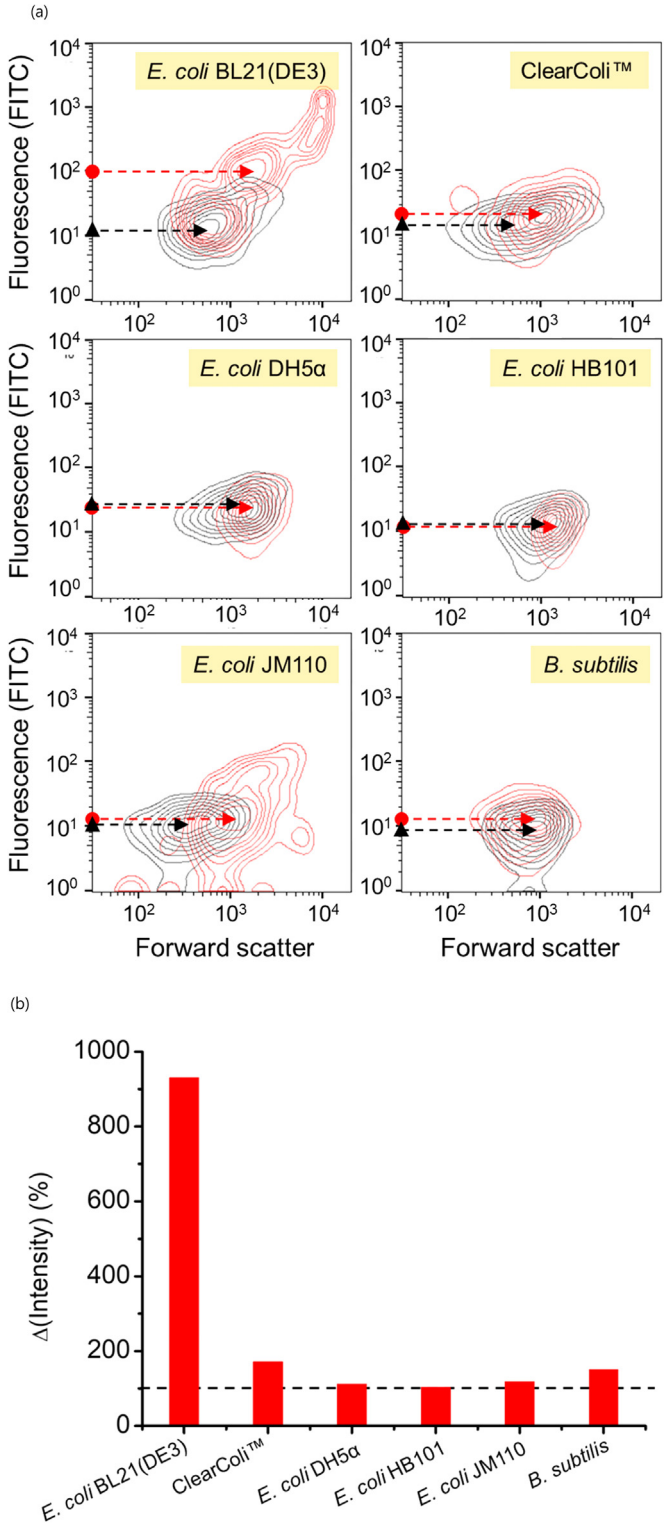

Fig. 5. Analysis of the specificity of anti-LPS antibodies separated from BL21(DE3). (a) Comparison of the FACS fluorescence signals before (black line) and after (red line) the treatment with anti-LPS antibodies for several bacterial strains, and (b) the intensity increment ratio for each strain.

labeled with fluorescence and FACS analysis. As shown in Fig. 4(b), the fluorescence of ClearColi (④ in Fig. 4(a)) was not significantly increased in comparison with the baseline level (③ in Fig. 4(a)). However, the fluorescence of BL21(DE3) (② in Fig. 4(a)) was significantly increased in comparison with the baseline level (① in Fig. 4(a)). As shown in Fig. 4(b), ClearColi reacted with the anti-LPS antibodies, showing

Table 1
Summary of the selectivity test with the separated anti-LPS antibodies by FACS analysis.

	***E. coli* BL21(DE3)**	**ClearColi™**	***E. coli* DH5α**
w/o anti-LPS Ab	11.6 (60.4[a])	19.2	23.2 (120.8[a])
w/ anti-LPS Ab	108 (327.3[a])	33	26.1 (79.1[a])
Increment	**931.00%**	**171.90%**	**112.50%**
	***E. coli* HB101**	***E. coli* JM110**	***B. subtilis***
w/o anti-LPS Ab	12.5 (65.1[a])	10.2 (53.1[a])	8.6 (44.8[a])
w/ anti-LPS Ab	12.9 (39.1[a])	12.1 (36.7[a])	13.0 (39.4[a])
Increment	**103.20%**	**118.60%**	**151.20%**

[a] Increament when compared with the intensity of ClearColi.

fluorescence levels of less than 10^2 (AU) for 99.9% and 99.8% of the total bacteria population before and after treatment with the anti-LPS antibodies, respectively. When BL21(DE3) was reacted with the separated anti-LPS antibodies, it showed a fluorescence level of less than 10^2 (AU) for 99% of the total BL21(DE3) population before the treatment with anti-LPS antibodies, and a level of greater than 10^2 (AU) for more than 66.2% of the total BL21(DE3) population after the treatment with anti-LPS antibodies. These results indicated that the separated anti-LPS antibodies could bind selectively to more than 66.2% of the total BL21(DE3) population.

The specificity of the anti-LPS antibodies separated from BL21(DE3) was quantitatively analyzed using other kinds of gram-negative bacteria (viz., *E. coli* strains DH5α, HB101, and JM110) and a gram-positive bacterium (*Bacillus subtilis*) by using FACS analysis. The bacterial strains were treated sequentially with the separated anti-LPS antibodies from human serum, and then with the bacterial strains were treated anti-hIgG labeled with fluorescence (FITC). The density plot was made before and after the treatment of antibodies. The size distribution of bacteria was estimated by forward scattering (x-axis) and the fluorescence level was estimated at the same time (y-axis). When anti-LPS antibodies were bound to the outer membrane of bacteria, the size distribution (x-axis) as well as the fluorescence level (y-axis) could be changed as shown in Fig. 5(a). As shown in Fig. 5(b), the binding of anti-LPS antibodies to each bacterial showed that BL21(DE3) made a significantly large difference in fluorescence from the analysis of density plots. These results indicated that the separated anti-LPS antibodies showed a far higher specificity toward BL21(DE3) than to the other *E. coli* strains and the gram-positive bacterium. The detailed parameter from FACS analysis of each strain was summarized in Table 1.

The dissociation parameter (K_d) of the separated anti-LPS antibodies was analyzed in solution phase by thermophoretic immunoassay. The principle of this assay was based on the difference in mobility between the antigen–antibody complex and the unbound antigens and antibodies under infrared radiation. Usually, antigens or antibodies were labeled with a fluorescent tag for the monitoring of their movement. In this work, the outer membrane of BL21(DE3) was prepared as liposomes with a diameter of 100 nm into which a fluorescent protein named tdTomato was inserted. Additionally, the outer membrane of ClearColi was also prepared as tdTomato-containing liposomes as a negative control. In this work, the dissociation constant was estimated for two types anti-LPS antibodies which were prepared in two different ways: (1) without protein-A separation, and (2) with protein-A separation. Anti-LPS antibodies from both types of preparations were submitted to thermophoretic immunoassay analysis, where the difference in the fluorescence intensities (by turning on and turning off the infrared radiation) was taken as a thermophoretic signal. The signals from the liposomes of BL21(DE3) and ClearColi were then plotted against a specific concentration range of anti-LPS antibodies. As shown in Fig. 6(a), anti-LPS antibody prepared without protein-A separation was applied to ClearColi and BL21(DE3). When the anti-LPS antibodies at different concentrations (0.128 and 33 μg/mL) were treated to both strains, the change in fluorescence signal was not so significantly

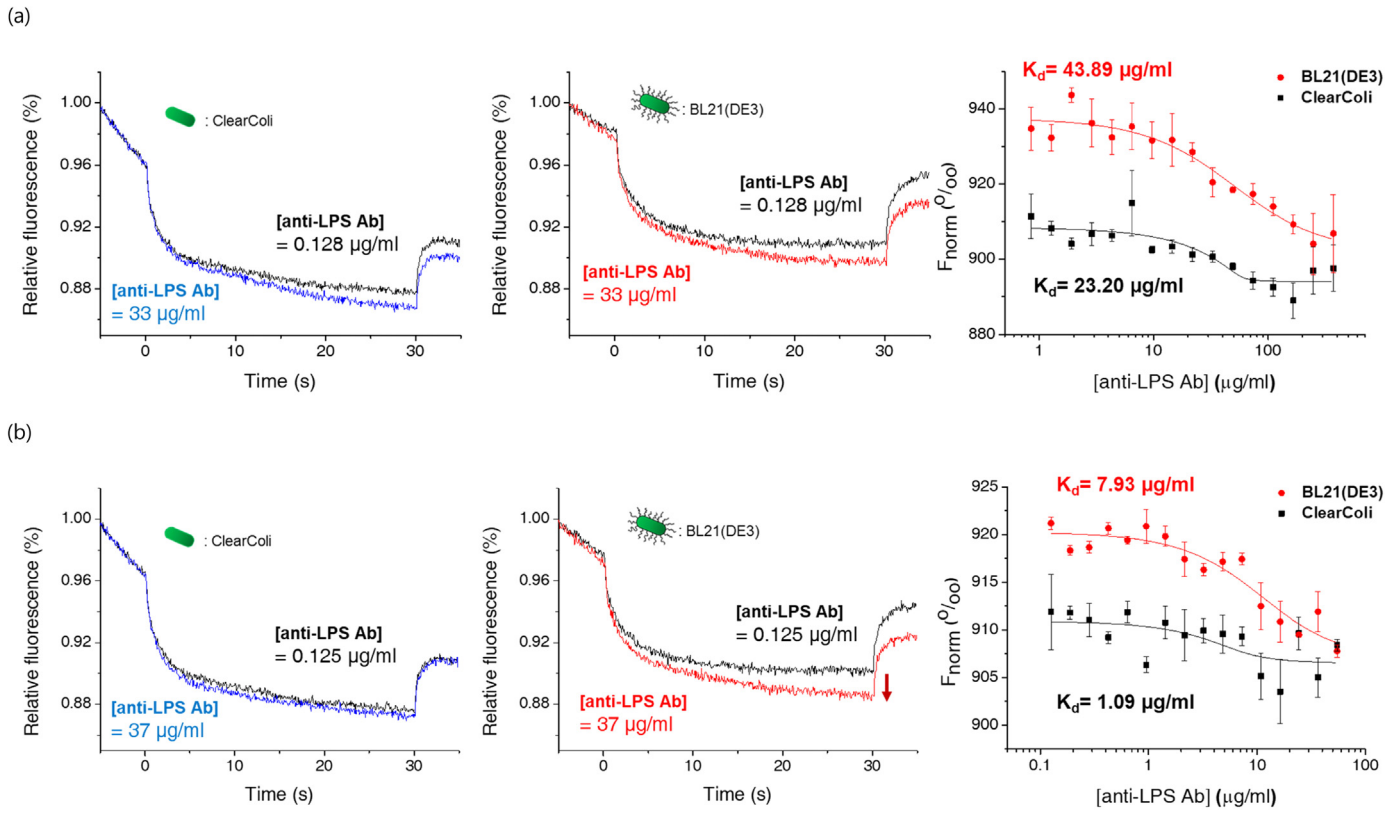

Fig. 6. Thermophoretic analysis of the binding constant of the separated anti-LPS antibodies. (a) Thermophoretic analysis of the binding of BL21(DE3) and ClearColi to the separated anti-LPS antibodies before protein-A purification. (b) Thermophoretic analysis of the binding of BL21(DE3) and ClearColi to the separated anti-LPS antibodies after protein-A purification.

different. However, the treatment of anti-LPS antibodies with protein-A separation at the concentration of 0.125 and 37 μg/mL with protein-A separation to ClearColi made nearly no change in fluorescence signal as shown in Fig. 6(b). When the anti-LPS antibodies at the same concentrations was treated to BL21(DE3), there occurred a significant change in the fluorescence signal. These results showed that the anti-LPS antibodies with protein-A purification could selectively bound to BL21(DE3) in comparison with those before protein-A purification. In order to quantify such a difference, the dissociation parameter (K_d) was estimated for the anti-LPS antibodies not purified with protein-A in the concentration range of 0.852–373 μg/mL, and the dissociation constant was estimated for BL21(DE3) and ClearColi to be were estimated to be 43.89 and 23.20 μg/mL, respectively (Fig. 6(a)). The dissociation constant (K_d) was estimated for the anti-LPS antibodies not purified with protein-A in the concentration range of 0.125 – 55 μg/mL, and the dissociation constant was estimated for BL21(DE3) and ClearColi to be were estimated to be 7.93 and 1.09 μg/mL, respectively (Fig. 6(b)). The great difference in the dissociation constants for BL21(DE3) after the protein-A purification showed that the binding activity of anti-LPS antibodies could be enhanced with protein-A purification. Additionally, the lower dissociation constant for ClearColi after protein-A separation than that before the column chromatography showed that the non-specific binding of anti-LPS antibodies could be decreased by the protein-A purification step. On the basis of these results, the protein-A separation step was always used in the separation protocol of anti-LPS antibodies from serum.

3.3. Fluorescence immunoassay with the separated anti-LPS antibodies

The binding properties of the separated anti-LPS antibodies were also tested after their immobilization onto solid supports. As shown in Fig. 7(a), the separated anti-LPS antibodies were immobilized onto a microplate, the surface of which had been modified with parylene-H for the covalent bonding of antibodies. BSA was used to prevent the non-specific binding of proteins to the active formyl groups of the parylene-H surface. GFP-expressing BL21(DE3) at the concentration range of 1.4×10^3 to 1×10^6 cells/mL was added to the modified plate surface on which anti-LPS antibodies had been immobilized. As shown in Fig. 7(b), BL21(DE3) had bound to the surface with anti-LPS antibodies, where the number of bound BL21(DE3) cells was in proportion to the initial concentrations of BL21(DE3). In the case of GFP-expressing ClearColi, its binding to the same modified surface with immobilized anti-LPS antibodies was observed to be insignificant in comparison with that of BL21(DE3), even though the cell concentration range was the same as that of BL21(DE3). The limit of detection (LOD) of fluorescence immunoassay was estimated to be three sigma (standard deviation) from mean vale of blank sample (Di Muccio et al., 2006). As shown in Fig. 7(c), the baseline for blank sample was estimated to be 1252.0 (AU) and the standard deviation was estimated to be 102.6 (AU). From these results, the LOD was calculated to 1.56×10^3 cells/mL and a limit of quantification (LOQ) of 5.2×10^3 cells/mL. From these results, the standard curve for the fluorescence immunoassay was made in the bacterial concentration range of 1.56×10^3 to 1×10^6 cells/mL (R^2 = 0.997) as shown in Fig. 7(c). These results showed that the binding response of BL21(DE3) was significantly higher than that of ClearColi for the entire dynamic concentration range. These results showed that the separated anti-LPS antibodies could be effectively used for the binding of BL21(DE3) after their immobilization.

To further verify their applicability, the separated anti-LPS antibodies were immobilized onto a gold electrode, and the impedance change was measured before and after the addition of BL21(DE3) and ClearColi (as a negative control). From the impedance measurements, Nyquist plots were drawn for the subsequent reaction steps of immobilization of the separated anti-LPS antibodies and blocking with

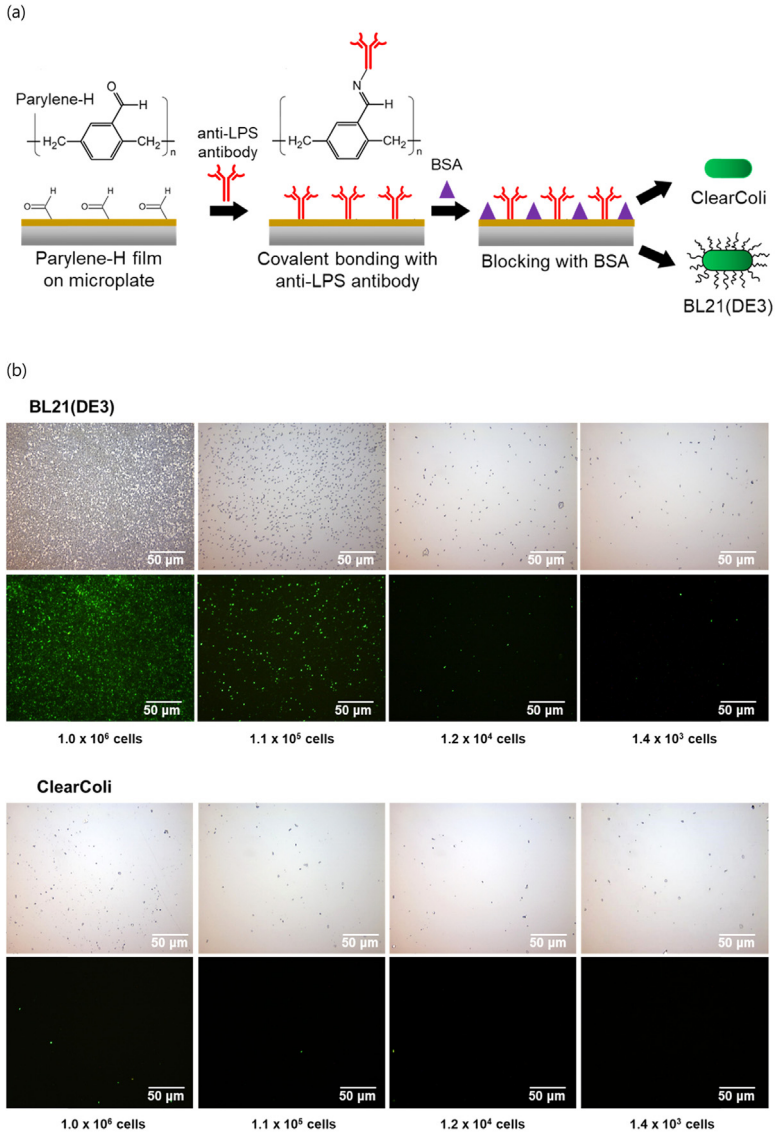

Fig. 7. Fluorescence immunoassay of BL21(DE3) with immobilized anti-LPS antibodies. (a) Schematic view of the modified surface of the microplate for the immobilization of the separated anti-LPS antibodies. (b) Fluorescence images of the binding of BL21(DE3) (upper images) and ClearColi (lower images) to the immobilized anti-LPS antibodies. (c) Standard curve for the fluorescence immunoassay of BL21(DE3).

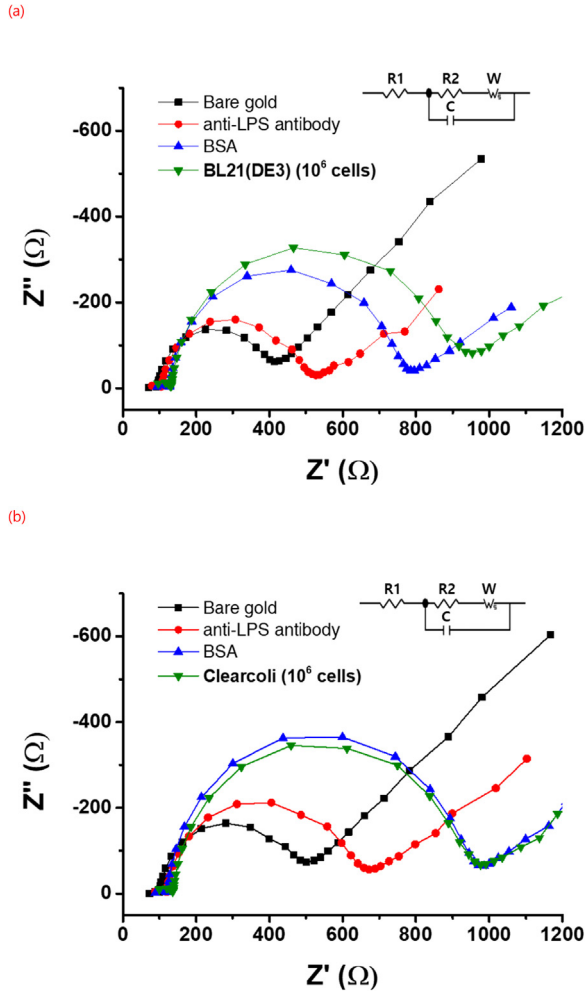

Fig. 8. Impedance analysis of the binding of BL21(DE3) to anti-LPS antibodies immobilized onto a gold electrode. From the impedance measurement, Nyquist plots were drawn for the subsequent reaction steps of binding of the anti-LPS antibodies, blocking with BSA, and then binding of (a) BL21(DE3) and (b) ClearColi to the antibody-immobilized gold electrodes.

BSA. Then, samples of BL21(DE3) or ClearColi were injected onto the gold electrodes with the immobilized anti-LPS antibodies. Nyquist plot was analyzed by modified Randle's model (Fig. 8(a), (b) inset) which includes solution resistance (R1), charge transfer resistance (R2) with parallel capacitance (C) and warburg impedance (W) (Abbasi et al., 2018; Benvidi et al., 2018; Ehret et al., 1997; Moraes et al., 2012; Nguyen et al., 2018; Park et al., 2018). As shown in Fig. 8(a), the charge transfer resistance (R_{ct}) increased from that for bare gold (R_{ct} = 304.2 Ω) to 475.4 Ω and 534.2 Ω after the binding of anti-LPS antibodies and the BSA blocking step, respectively. When BL21(DE3) was added, the R_{ct} value changed to 809.3 Ω as a result of BL21(DE3) cells binding to the electrode surface. However, there was no significant change in the R_{ct} value after the addition of ClearColi (Fig. 8(b)) because ClearColi could not bind to the anti-LPS antibodies immobilized on the gold electrode. These results showed that the separated anti-LPS antibodies could be effectively used for the detection of BL21(DE3) (or bacteria with LPS) after their immobilization onto a gold electrode.

4. Conclusions

In this work, the gram-negative bacterium *E. coli* BL21(DE3) (with LPS in its outer membrane) and its modified ClearColi (without LPS) were used for the separation of anti-LPS antibodies from human serum. The anti-LPS antibodies in the serum were separated by the following steps: (1) binding of serum proteins to BL21(DE3); (2) dissociation of the bound proteins; (3) filtering with ClearColi; and (4) separation of the antibodies with protein-A column chromatography. The optimal dissociation condition was determined by FACS analysis to be the treatment of an acidic solution (0.1 M glycine-HCl, pH 2.7) for 10 s. Additionally, the optimal filtering process with ClearColi was determined to be less than three times. The yield of anti-LPS antibodies after protein-A column separation was determined to be 10.23 ± 0.53% (n = 5). The absolute amount of anti-LPS antibodies from 1 mL of human serum was 0.0231 ± 0.007 mg (n = 18). The binding property of the separated antibodies was analyzed by FACS and thermophoretic immunoassay. According to the FACS analysis, the separated anti-LPS antibodies could bind selectively to more than 66.2% of BL21(DE3) cells among the total population. According to the thermophoretic signals, the dissociation constants (K_d) of anti-LPS antibodies for the outer membranes of BL21(DE3) and ClearColi were 7.93 and 1.09 μg/mL, respectively. As the next step, the applicability of the separated anti-LPS antibodies to the in vitro testing of gram-negative bacteria was demonstrated after immobilization of the purified antibodies onto different solid supports. The fluorescence immunoassay was carried out at the bacterial concentration range of 1.56×10^3 to 1×10^6 cells/mL of BL21(DE3). The binding response was observed to be significantly higher than that of ClearColi for the entire dynamic concentration range. Moreover, the impedance change on gold was measured before and after the addition of BL21(DE3) and ClearColi (as a negative control) to the antibodies immobilized on the electrode, where the R_{ct} was observed to have increased to 809.3 Ω owing to the binding of BL21(DE3) to the electrode surface. However, there was no significant change in the R_{ct} after the addition of ClearColi. These results showed that the separated anti-LPS antibodies could be effectively used for the detection of BL21(DE3) (and other bacteria with LPS) after their immobilization onto a gold electrode.

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