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PII: S0731-7085(17)31544-3
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2017.07.043
Reference: PBA 11423

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 14-6-2017
Revised date: 28-7-2017
Accepted date: 29-7-2017

Please cite this article as: Min Park, Jae-Chul Pyun, Joachim Jose, Orientation and density control of proteins on solid matters by outer membrane coating: analytical and diagnostic applications, Journal of Pharmaceutical and Biomedical Analysishttp://dx.doi.org/10.1016/j.jpba.2017.07.043

# Orientation and density control of proteins on solid matters by outer membrane coating: analytical and diagnostic applications

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Highlights

- A lack of orientation control exists when solid matters are coated with biomolecules
- This leads to a severe reduction in sensitivity and the limit of detection
- Autodisplay leads to an oriented expression of proteins on the OM
- OM liposomes can be prepared and used for solid matter coating
- Orientation and density control obtained thereby leads to an increase in sensitivity and LOD

**Abstract**

Autodisplay is an expression system for the display of recombinant proteins on the outer membrane (OM) of gram negative bacteria and has been developed for translocation studies, whole cell biocatalysis, bioremediation, inhibitor screening, and enzyme refolding. Recently, affinity proteins such as IgG-binding Z-domains and biotin-binding streptavidin have been autodisplayed on the OM of *Escherichia coli* for analytical and biomedical applications. The secretion mechanism of the autodisplay system was used and orientation and density control of these affinity proteins were determined. Affinity protein-autodisplaying *E. coli* cells have been used to coat solid supports in immunoassays. For this purpose, the OM of autodisplayed *E. coli* cells was separated and isolated by the aid of detergents. The structure of the resulting OM liposomes as well as their physico-chemical parameters, were analyzed. OM liposomes were used subsequently for coating various solid matters including microplates and biosensor transducer surfaces and the formation of OM layers were monitored. OM layer formation on solid matters was shown to increase the sensitivity of immunoassays and biosensors. In this review, analytical and diagnostic applications are described in particular concerning orientation and density control of autodisplayed affinity proteins.

**Key words**: autodisplay, affinity proteins, immunoaffinity layer, orientation control, density control, medical diagnosis

## 1. Introduction

Immunoassays and biosensors have been applied for medical diagnosis to detect infectious agents, such as viruses and bacteria, and the products of self-defense mechanisms of the human body, such as antibodies and cancer markers [1]. Enzyme-linked immunosorbent assay (ELISA) is one of the most frequently used immunoassays for medical diagnosis [2]. Among the several ELISA methods such as direct, indirect, sandwich, and competitive ELISA, sandwich ELISA is the most widely used because of its sensitivity and availability. In this method, analyte-binding antibodies (capture antibodies) are coated onto a solid support such as 96-well polystyrene microplates. After analyte addition and binding to the capture antibody layer, enzyme-labeled

antibodies are added for detection. The analyte is quantified by a chromogenic reaction, luminescence, or fluorescence produced from the enzyme-labeled antibody. ELISA is widely used for immunoassays because of its excellent limit of detection. However, this method requires a relatively long incubation time for analyte binding and antibody detection. For biosensors, the immobilization of antibodies to the surface of the transducer is essential for target analyte recognition [3].

For detection in the sandwich type ELISA, the analyte should be bound to immobilized capture antibodies. Therefore, the sensitivity of this ELISA method is directly related to the number of exposed antigen binding sites for the capture antibodies. It is well-known that antibodies have a 'Y' shaped symmetric structure. An antibody has three branches, and the antigen-binding site, the Fab region, are small and localized at the two ends of the antibody's branch. Because of this structural reason, it has been reported that antibody layers prepared by physical adsorption or chemical coupling result in randomly oriented antibodies, where only 20 % of antibodies have proper orientation for binding analytes [4]. Therefore, effective detection of a target analyte by the antibodies at the immunoaffinity layer is possible when the Fab regions of the antibodies are exposed toward the analyte by orientation control methods [5, 6]. The density of antibodies on the immunoaffinity layer should also be controlled to improve immunosensor sensitivity. Thus, analyte binding sites need to be maximized in relation to the number of immobilized antibodies.

As mentioned above, in order to obtain sensitive immunoassays and biosensors, immobilization of antibodies on a solid support is essential. Using classical methods, antibodies have been immobilized by simple physical adsorption in random orientation. Chemical methods for immobilizing antibodies by using functional groups through self-assembled monolayers (SAMs) are also widely used. For the enzyme immobilization with orientation control, click chemistry was used by Guan et al [7] and enzyme autodisplaying system was applied for the attachment of bacteria cell to the electrode surface [8]. However, antibody orientation cannot be controlled when using such physical and chemical immobilization methods. Therefore, various methods for immobilizing antibodies in a specific orientation have been developed [9]. These studies have focused on immobilization of the Fc region of the antibodies to expose the antigen-binding sites of such antibodies [3]. Specific IgG binding proteins such as protein A, protein G, and protein L have been used to immobilize the capture antibody *via* non-covalent binding. These affinity proteins can easily immobilize antibodies in a controlled orientation, but it is worth mentioning at this point, that they also require previous immobilization on

the solid matter, if possible in an orientation controlled manner. In this review, various applications of immunoassays and biosensors based on the autodisplay of affinity proteins, as such enabling orientation and density control, are presented and discussed with respect to their activa and their passiva..

**2. Autodisplay is using the autotransporter secretion pathway: orientation control is mechanism based**

Autodisplay technology was developed on the secretion mechanism of the autotransporter family of proteins [10-13]. The first autotransporter was immunoglobulin A1 (IgA1) protease found in *Neisseria gonorrhoeae* in the 1980s [14-17]. Unlike IgA1 protease, a naturally existing autotransporter, named as ***a**dhesin **i**nvolved in **d**iffuse **a**dherence* (AIDA-I), was found in *Escherichia coli* [11, 18]. The term autodisplay originally referred only to applications using the AIDA-I autotransporter domain [11, 13]. Recently, however, autodisplay has come to include recombinant expression by all other autotransporter proteins that can pass through the inner membrane (IM) without artificial treatment and become anchored within the outer membrane (OM) of gram-negative bacteria by a β-barrel [13]. The autodisplay system was developed according to the type V secretion mechanism; in particularly according to the type Va mechanism, as a subtype of type V [11, 19]. As shown in Fig. 1, a typical signal peptide (SP) is located at the N-terminus and the sequence of the target cargo is located between the SP sequence and the linker domain of the original AIDA-I protein encoded by appropriate plasmids. Subsequent to the linker domain, a β-barrel domain follows, which is exploited to transport the cargo to the cell surface and serves afterwards for OM anchoring. The expressed recombinant passenger protein, or cargo, is first transported from the cytosol to the IM. Next, the protein passes through the IM by the Sec pathway. For this purpose, the signal peptide (SP) of the β-subunit of cholera toxin from *Vibrio cholerae* was used most frequently. Transport across the IM results in the localization of the cargo in the periplasm (PP). Arriving in the PP, the β-barrel domain forms a porin-like structure by interacting with the OM. Using the α-helix containing linker domain, that passes the lumen of the β-barrel structure, the target cargo can be displayed on the outside of the bacterial cell [17, 18, 20, 21]. Autodisplayed cargo or target proteins are C-terminally attached to the α-helix containing linker domain and hence - as long as the secretion mechanism is working - are displayed in an identical orientation directed to the cell surface [22]. The autodisplay technology was first used to express various enzymes [23]. Cells displaying these enzymes were applied in translocation studies, whole cell

biocatalysis, bioremediation, inhibitor screening, and enzyme refolding [24-29]. By these studies it turned out that an approximate number of n x $10^5$ molecules can be displayed on a single cell. For bovine adrenodoxin, an electron transfer protein, a number of 1,8 x $10^5$ per single cell was determined by its electron transferring capacity. Subsequent estimations lead to the conclusion that by applying four times this number, the outer membrane would be completely filled up with β-barrel domains [11]. Hence, autodisplay was supposed to yield a high density of the protein expressed by this mechanism within the outer membrane.

---------- Fig. 1 ----------

To control the orientation and density of antibodies for detection purposes using the autodisplay technology, affinity proteins have been displayed. Among affinity proteins for IgG type antibodies, protein A, protein G, and protein L are the most widely used [30-32]. Protein A is a cell-wall-bound pathogenicity factor from *Staphylococcus aureus* [32]. The molecular weight of protein A is 42 kDa according to sedimentation equilibrium analysis and consists of 31% α-helix, 13% β-structure, and 56% random coil [33, 34]. This protein has a markedly extended shape with an average Stoke's radius of 5.0 nm [33, 34]. Protein A shows immunoglobulin (Ig)-binding activity, particularly with IgG, IgA, and IgM. The binding site between Ig and protein A is not an antigen co-binding site, but rather resembles a Fc domain, consisting of a tandem repeat of five highly homogeneous IgG-binding domains: E, D, A, B, and C, with approximately 58 amino acid each [35]. The dissociation constant of protein A is 4.8 × $10^7$ M with human polyclonal IgG and 4.1 × $10^8$ M with rabbit polyclonal IgG [36-38]. Because protein A has specific binding affinity for the Fc region of IgG, it is commercially used for antibody immobilization and purification [39-41]. More recently, the Z-domain derived from protein A has been used for similar purposes. The Z-domain is an engineered analogue of the B domain derived from protein A. It was originally developed as an affinity-purification tag for fusion protein production [42]. The Z-domain was shown to oligopolymerize, either to form a dimer (ZZ-domain) or a tetramer (ZZZZ-domain) to improve antibody affinity [42]. A single Z domain consists of three α-helices and binds the Fc region of the antibody *via* helices 1 and 2 by hydrogen bonding [42, 43].

Avidin and streptavidin are two additional affinity proteins, commonly used in biomedical applications. Avidin is a 66–69-kDa tetrameric protein found in the egg white of birds, reptiles, and amphibians [44-46]. Streptavidin is found in *Streptomyces avidinii,* and both avidin and streptavidin show affinity for biotin, also known as

vitamin B7 or vitamin H [44, 45]. Streptavidin is a tetrameric protein with eight antiparallel β-strands and each subunit contains a biotin-binding site [47, 48]. Based on thise structural consideration, a monomeric streptavidin was developed with similar binding affinity to biotin [49, 50]. The dissociation constant of the streptavidin and biotin interaction is $10^{13}$–$10^{16}$ M and shows the highest affinity among naturally occurring non-covalent bonds [45]. This interaction is mediated not only by hydrogen bonds, but also included a substantial portion of hydrophobic interactions [48]. This strong binding is pseudo-irreversible as well as durable in the presence of organic solvents, detergents, pH, and high temperature. To exploit the strong bonding between biotin-streptavidin, various biotinylation strategies were developed [51-54]. Streptavidin had been widely applied for immunoassay and biosensor development [55-58]. For example, a streptavidin layer can be used to immobilize a biotinylated antigen, and subsequently antigen specific antibodies can be detected as analytes. In consequence, streptavidin coating has been applied for the diagnosis of autoimmune diseases or in anti-virus antibody testing.

For similar purposes, the Z-domain as mentioned above and streptavidin were displayed on the OM of *E. coli* using autodisplay [59, 60]. As shown in Fig. 1(a), the Z-domain encoding sequence was inserted between the SP and the linker and β-barrel encoding domains at the XhoI and KpnI restriction sites [59, 61, 62]. After transformation of the corresponding Z-domain autotransporter fusion protein encoding plasmid into *E. coli* cells, the expressed Z domain was transported through the IM by the SP and was displayed on the OM surface by the folded β-barrel [63]. By densitometry analysis, the intensity of the band representing the expressed Z protein after SDS-PAGE was compared with the band intensity of OmpA, a natural occurring OM protein with a constant number of $10^5$ molecules per cell [59, 64]. By comparing the band intensities and putting them into relation with the corresponding molecular weights of the to proteins, the number of autodisplayed Z-domain and streptavidin was determined to be $2.8 \times 10^5$ and $1.6 \times 10^5$ molecules per cell, respectively [59, 60]. Hence, density of a protein displayed at the cell surface e.g. the Z-domain or streptavidin can be controlled by the promotor used for expression of the recombinant protein by autodisplay [22].

By autodisplay of affinity proteins such as the Z-domain or streptavidin, a molecular recognition layer can be created (Fig. 2a) [65-67]. Autodisplayed Z-domains can be used to control the orientation of antibodies by taking advantage of their Fc region binding properties [39, 42]. When capture antibodies were incubated with autodisplay Z-domains, their Fc region was immobilized and antigen-binding sites were exposed towards the analyte by the mechanism-based orientation control (Fig. 2b). This led to a substantial increase in the sensitivity

of immunoassays as well as of biosensor based detection. Autodisplayed streptavidin could also be used to form an affinity layer for strong binding of biotin [60, 68]. When biotinylated antibodies were used to treat monolayers obtained by OM vesicles obtained with autodisplayed streptavidin, similar orientation effects were observed, but showing higher binding affinities than obtained with autodisplayed Z-domains. Furthermore, autodisplayed streptavidin could be used to form an affinity layer for the detection of antibodies as analytes by immobilizing biotinylated antigens.

---------- Fig. 2 ----------

An exceptional feature of autodisplay is, that the anchoring domain of the passenger the so-called β-barrel is no covalently linked to the cell envelope, but remains motile. This is due to the physico-chemical properties of the β-barrel, which has a belt of hydrophobic amino acids at the outside directed towards the membrane and hydrophilic amino acids at the inner side forming the pore for the passenger and the linker domain (Fig. 2b). By comparing the electron transfer activity of surface displayed adrenodoxine (Adx) with the activity of free molecules, a number of $1.8 \times 10^5$ molecules per single cell was determined [25]. For sorbit dehydrogenase (SDH) the number of surface displayed molecules with autodisplay was determined to be $3 \times 10^5$ by fluorescence labeling and flow cytometry [69]. For Adx expression the strong T7/lac promotor was used and an equal distribution over the entire cell surface could be shown [13]. With a diameter of 1.1 nm the anchoring β-barrel was calculated to fit with a maximum number of $3.6 \times 10^5$ copies into the cell envelope, which is containing also the β-barrel proteins OmpA and OmpC/F to a similar extent [13, 70]. This is reflecting quite well the number of autodisplayed proteins as determined experimentally. When a weaker constitutive promotor was used [21] or a titratable promotor such as the rhamnose promotor [71], the autodisplayed proteins still appeared equally distributed over the cell surface, but now in lower numbers, e.g. $1 \times 10^4$ [71]. This is indicating that, by the choice of the promotor, the density of the molecules within the outer membrane can be controlled with a maximum in the range of $3.6 \times 10^5$ and a minimum depending on the promotor's strength. Hence, the autodisplay mechanism not only allows the control of orientation of proteins displayed in the outer membrane, but also the control of density by use of an adequate promotor.

**3. Shape of the OM and physico-chemical parameters of OM vesicles as obtained by autodisplay**

Bacteria are classified as gram-positive or gram-negative by a staining according to the procedure of late Christian Gram. The autodisplay technology can be used only in gram-negative bacteria including *E. coli*. The cell envelope of gram-negative bacteria consists of the OM, a single peptidoglycan layer, and the IM, as seen from the outside to the inside of a cell [73]. IM encapsulates the cytosol of *E. coli* and the PP is located between the OM and IM. The PP contains a rigid peptidoglycan monolayer.The OM of *E. coli* consists of lipopolysaccharide (LPS), lipoproteins, phospholipids, other OM proteins, such as OmpA, OmpC, and OmpF and has a thickness of 7 nm [74, 75]. Lipoproteins and OmpA in the OM binds to the peptidoglycan monolayer to form an expected 5–7 nm lumen between the two membrane layers, IM and OM [76]. *Escherichia coli* cells with autodisplayed affinity proteins can be used to coat solid supports for immunoassays. [60-62, 66, 67]. Due to the cylindrical shape of *E. coli* cells, with 1 µm in width and 2 – 5 µm in length, sensoring and signal recording is difficult when whole cells are used for coating with evanescence field-based biosensors [61, 62]. Nevertheless, whole cells of *E. coli*, displaying proteins on the surface have been used for analytical purposes [60, 62, 77]. To overcome the limitations related to the size of *E. coli* cells, the OM of *E. coli* cells was isolated and formed a layer on solid matter [64, 65]. In this respect, isolation of cell membranes from various bacterial cells including *E. coli* has been extensively studied. In most examples amphiphilic molecules such as detergents were used to solubilize membrane proteins or to isolate the membrane with the included proteins as a kind of vesicle [78-82]. Filip et al. [79] used sodium lauryl sarcosinate and Schnaitman [80] used Triton X-100 to isolate the IM from *E. coli* cells. In another study, OM vesicles, which are naturally produced by *E. coli* cells for cellular signaling and which are also called “secreted bacterial outer membrane vesicles” were used for immobilization of OM proteins. Finally, an artificial phospholipid bilayer has been used for similar purposes [83]. For *E. coli* OM, an isolation method introduced by Hantke and modified by Schultheiss et al. has been used [84, 85]. In this method Triton X-100 and lysozyme were applied for OM isolation [86]. After cultivation and induction of the target protein-expression and surface display, bacterial cells were suspended in Tris-HCl buffer pH 8,0. Next, sucrose was added for osmosis and EDTA was added to increase permeability by removal of $Ca^{2+}$ or $Mg^{2+}$ ion binding to LPS of the OM. The rigid peptidoglycan layer in the PP was hydrolyzed by the addition of lysozyme. After cell wall hydrolysis, *E. coli* cells lose their shape and form rotund spheroplasts. Protease inhibitors, including phenylmethylsulfonyl fluoride and aprotinin, are added to the spheroplasts to prevent OM proteins from being digested by proteases of the PP during the procedure. Next, the OM is isolated

as a kind of vesicle by treatment with extraction buffer containing DNases and Triton X-100 [86]. Isolated OM vesicles are purified by a several centrifugation and washing steps. As an alternative, Bond et al. [85] have developed an enzyme-free OM isolation method involving ultrasonification, but with all other steps similar to the method described above [87]. In contrast to Hantke's protocol, however, *E. coli* cells are directly added to the extraction buffer without sucrose, EDTA, or lysozyme treatment. The rigid peptidoglycan layer is disassembled by ultrasonic treatment rather than by enzymatic hydrolysis. After 120 cycles of ultrasonification, isolated OM vesicles are purified in by several centrifugation and washing steps again.

For purity analysis, isolated OMs were qualitatively and quantitatively analyzed by western blotting and enzymatic assays, respectively. As shown in Table 1, after disruption of *E. coli* cells four fractions were obtained, OM, PP, IM, and the cytoplasmic fraction, and for each fraction, a characteristic marker protein was selected. For OM, OmpA was chosen and showed a clear and distinct band in SDS-PAGE analysis. Western blotting analysis showed that the isolated OM did not contain any PP, IM, or cytoplasm marker, while the whole cell lysate showed all proteins. A quantitative enzymatic assay of the selected markers showed that the isolated OM contained less than 1% impurities from the IM, PP, and cytoplasm. Thus, OM can be isolated with high purity by using lysozyme and Triton X-100. The OM structure is composed of a phospholipid bilayer that can theoretically form two types of particles by isolation with Triton X-100: micelles and liposomes [88-90]. When the OM preparation was analyzed by a particle size analyzer, the size of OM particles was found to be approximately 100 nm [87, 91]. Because this particle size is too large to be considered a micelle, the isolated outer membrane needs to form liposomes [92]. In conclusion, the OM of *E. coli* can be isolated as a liposome with high purity, and OM preparations can be applied to determine the density of autodisplayed proteins or layering for medical diagnosis and biosensor applications [59, 61, 62, 66].

**4. Physico-chemical requirement of solid matters that can be coated with OM vesicles**

Planar coating technology in which OM liposomes were laid onto a two-dimensional substrate has been used to study of transmembrane proteins and immobilize biomolecules such as peptides, proteins, and enzymes [99-101]. To form a planar lipid bilayer, various layering methods have been developed, such as the Langmuir-Blodgett

(LB) film, which was shown to form a monolayer by using amphiphilic molecules [102, 103]. Using this classical LB film, the lipid can be transferred to a hydrophobic substrate i.e. the solid support [104, 105]. SAMs (self assembled monolayers), which thermodynamically align on the surface of the solid support, can immobilize biomolecules such as proteins, cells, and DNA through covalent bonding [106]. Mun and Choi produced a biotin-immobilized hybrid bilayer using SAM and applied this bilayer for biosensing of avidin [107]. For the OM of autodisplayed *E. coli*, however, it is isolated as a liposome and has a limited ability to form a layer as shown with SAM or with the LB film. Liposomes are rather vesicles in which the aqueous volume is entirely enclosed by a membrane composed of lipid molecules and form a spontaneous bilayer on glass substrates by simple dipping [100, 108]. Cho et al. formed a liposome layer with liposomes that had been ruptured by heat application [99]. Keller and Kasemo used a quartz crystal microbalance to weight the liposome layer and found that liposomes underwent monolayer adsorption, bilayer adsorption, and intact vesicle adsorption on extreme hydrophobic surfaces, $SiO_2$ surfaces, and other hydrophilic surfaces, respectively [109]. Like described for the synthetic liposome coating, OM preparations were used to coat the solid substrate by simple dipping and hence immobilized the autodisplayed proteins simultaneously [60-62, 66]. The outwards directed side of the *E. coli* OM consists of LPS and has a negative charge. The inside part of the OM, which – in intact cells - contacts the PP, is covered by lipoproteins and turned out to be more hydrophobic than the outside. [110]. When OM preparations were used to treat solid support as substrates with different contact angles indicating different hydrophobic surface values, the coating density increased with increasing surface substrate hydrophobicity (Fig. 3 (a)) [62, 66]. Furthermore, the OM bilayer coated on the solid matter including the affinity proteins showed a very low level of non-specific binding, without the need for additional blocking steps such as bovine serum albumin treatment [62, 67]. For coating a solid support with OM preperartions, 300 µg/mL protein turned out to be saturating. This is reflecting the rather constant rate of protein content in the OM as obtained by autodisplay [61, 62]. Regarding coating kinetics, 120 min of incubation time was required to coat a microplate well [61]. By simple dipping, the OM formed a uniform layer on solid matter that was approximately 3.5 nm thick. When the OM layer on a gold substrate was analyzed by cyclic voltammetry to examine the redox reaction of ferricyanide, the total charge transfer was 5.4-fold lower than on bare gold (Fig. 3 (b) and (c)) [61]. When the OM layer was measured by impedance spectroscopy, the Rct value was 2.0-fold higher than that on bare gold (Fig. 3 (d)) [61]. Thus, a uniform electric isolation barrier was formed by coating the solid matter with

the isolated OM liposome from *E. coli* cells containing autodisplayed proteins.

---------- Fig. 3 ----------

Next important requirement, after formation of a uniform layer on the solid support is, that the activity of the autodisplayed protein is maintained, which is indeed the most important feature for diagnostic applications. The activity of the autodisplayed proteins within the OM based layer was analyzed by a variety of methods. Fluorescein-conjugated antibodies were added to the OM layer on gold substrate with autodisplayed Z-domains [66]. The fluorescence activity was measured with a fluorescence scanner and the OM layer exhibited a spatially uniform fluorescence. On OM layers without autodisplayed Z-domain, no fluorescence was observed. When the fluorescence intensity was quantitatively measured using a spectrophotometer, the OM layer with autodisplayed Z-domains showed a 3-fold higher intensity than the OM layer without Z-domains [87]. Based on these results, the IgG-binding activity of autodisplayed Z-domains was maintained after formation of a layer on solid matter without non-specific binding. As shown in Fig. 4, during the sequential treatment with different antibodies, morphology of the solid surface was followed by atomic force microscopy [61, 66]. The morphology changed by binding of antibodies to the autodisplayed Z-domains in the OM layer. In addition, the Rct value of the OM layer with autodisplayed Z-domains was increased 1.3 fold after antibody treatment [61]. Thus, IgG antibodies were immobilized on the OM layer with autodisplayed Z-domains after coating the solid matter. As described above, the OM with autodisplayed proteins yielded liposomes by the preparation process and formed a uniform layer on the solid support in a simple dipping step. After layer formation, the biological activity of the autodisplayed proteins were maintained. Thus, an affinity layer can be formed by OM liposomes containing autodisplayed affinity proteins, which could be subsequently utilized for medical diagnosis and biosensor purposes.

---------- Fig. 4 ----------

**5. Examples of applications**

OM preparations were coated onto a microplate or surface plasmon resonance (SPR) biosensor using a simple dipping method for applications in immunoassays and as immunosensors [61, 62, 66]. A microplate-based

immunoassay was developed based on the immunoaffinity layer of autodisplayed Z-domains [62]. As shown in table 2, OM coated microplate immunoassay showed 1.5 ng/mL of LOD. In contrast, immunoassay based on randomly oriented antibodies showed an LOD of 25 ng/mL. This microplate-based immunoassay showed a 30-fold improved LOD and 15-fold improved LOD, in comparison to physical adsorption, when used for the detection of horseradish peroxidase and CRP, respectively. These results reflect the orientation and density control of Z-domains, which have been autodisplayed and isolated within the *E. coli* OM preparation. The autodisplay-based immunoaffinity layer has also been applied for SPR biosensors [61, 66]. SPR biosensor is optimal for the application of OM layer because (1) SPR biosensor is optical biosensor without any external stimulus which can affect to the OM layer and (2) gold chip is the most widely used for the SPR biosensor. As described above, hydrophobicity of solid support is important factor for the formation of OM layer and gold is hydrophobic enough. From these reasons, SPR biosensor was selected for the application of OM based immunoaffinity layer. As SPR biosensors are evanescence field-based biosensors, the thickness of the sensitive layer is typically less than a few hundred nanometers [111]. Thus, the molecular recognition layer should be much thinner than the sensitive layer for the detection of analytes. Because *E. coli* cells are larger than this layer, OM vesicle based coating methods can be used instead for the application with SPR biosensors.

After adding the OM preparation to the surface of the gold chip of an SPR biosensor, capture antibodies were added to the OM layer with autodisplayed Z-domains. As shown in Fig. 5, during each step of this procedure, the SPR biosensor signal increased. This indicated that OM particles were coating the surface of the SPR biosensor and subsequently capture antibodies were bound by the autodisplayed Z-domains. This confirmed the formation of the immunoaffinity layer on the SPR biosensor. CRP as well as human IgG could be quantified as analytes using such immunoaffinity layers on SPR biosensor chips. The results obtained indicated 100-fold (CRP) and 10-fold (IgG) improved LOD values compared to the physical adsorption, by which the corresponding antibody is directly added to the gold surface. Thus, the sensitivity of the SPR biosensor was increased by the orientation and density control effect of the autodisplay based OM vesicle coating. Moreover, it was possible to regenerate this immunoaffinity layer by cross-linking after treatment with glutaraldehyde [61]. This treatment covalently linked the antibody to the autodisplayed Z-domain, giving resistance to low pH treatment, which could be used for antigen elution.

---------- Fig. 5 ----------

Streptavidin, as described above as a second example for an affinity protein, was autodisplayed by similar means as the Z-domain on the OM of *E. coli* by Park et al. [60]. The binding ability of autodisplayed streptavidin to biotinylated biomolecules was compared to that of magnetic beads with covalently immobilized streptavidin. Streptavidin was cross-linked with the amine functionalized magnetic beads by glutaraldehyde. Streptavidin-autodisplaying *E. coli* cells showed 1.3 fold higher saturation signal than magnetic beads. Thus, the density of autodisplayed streptavidin was far higher than that of streptavidin covalently immobilized to the magnetic beads. From this results obtained, density control of streptavidin autodisplaying *E. coli* was verified. The OM of *E. coli* cells with autodisplayed streptavidin was isolated and layered on the bottom of a microplate well. The binding affinity towards a biotinylated horseradish peroxidase was compared with that of streptavidin after physical adsorption to the microplate well. The sensitivity of the OM-coated microplate-based immunoassay were 100-fold lower than the physical adsorption and showed much broader dynamic range than the physical adsorption. These results demonstrate that orientation and density control of autodisplayed streptavidin indeed increased immunoassay sensitivity.

**6. Perspectives**

In this review, (1) autodisplay of affinity proteins such as Z-domains of protein A and streptavidin, (2) isolation of the OM of autodisplayed *E. coli* cells using detergent, (3) layering of the isolated OM on solid matters, (4) immunoaffinity layering using the coated OM of *E. coli* cells, and (5) applications in immunoassays and biosensors were summarized. Through the orientation and density control in the affinity layers based on the autodisplay technology, the sensitivity of immunoassays and biosensors could have been improved. In addition to the Z-domain and streptavidin, various affinity proteins such as protein G and protein L could be used as well. Protein G is a cell wall-binding protein found in group C (58 kDa) and group G (65 kDa) of streptococci [115-117]. Protein G contains three IgG-binding domains, two spacing groups, and one cell wall-binding domain [118]. Protein G also has specific Fc region binding affinity with a higher IgG binding affinity constant than protein A [118, 119]. Unlike protein A, protein G shows binding affinity to albumin and all human IgG subclasses including human IgG3, rat IgG, and goat IgG [115, 120, 121]. Using these IgG binding properties,

protein G has been applied in antibody affinity purification, immunoassays, and biosensors [122-124]. Protein A shows poor binding affinities to the IgG of some commonly used laboratory animals such as goat and rat. Protein G can be used to overcome the disadvantages of protein A. By autodisplay of protein G, wider applications to immunoassays and immunosensors are expected to be developed. Additionally, chimeric protein AG, which is synthesized using both protein A and protein G, is a strong candidate for application in autodisplay [125]. Protein L is 95 kDa and can be isolated from the anaerobic bacteria *Peptostreptococcus magnus* [126]. In contrast to protein A and protein G, protein L does not show Fc region binding affinity, but has affinity to the Fab region of the light chain [126, 127]. Based on this binding property, protein L has been applied to immunoassays, biosensors, and purification of various Ig proteins such as IgG, IgA, and IgM [128, 129]. The use of protein L is limited because it can bind with only the kappa chain among the light chains; however, this may be advantageous when using antibody fragments such as single-chain Fv fragment (scFv) [128, 130]. Thus, autodisplay of protein L is expected to control the orientation and density of the Fab fragment or scFv. Coating of the OM with these autodisplayed affinity proteins may increase the sensitivity of the immunoaffinity layer.

Using the autodisplay technology, target proteins can be anchored to the OM through an α-helix containing linker connected to an OM anchoring β-barrel [13, 20]. The C-terminus of target proteins are connected to the linker on the OM so that all autodisplayed proteins are expressed in same orientation. Using this orientation of autodisplay system, direct expression of antibodies or their libraries can be used for orientation and density control in immunoassays and biosensors. Various surface display strategies for peptide or antibody libraries on phage, bacteria, and yeast have been studied for drug screening and other applications [131-133]. For the autodisplay of antibodies, a peptide library or scFv library rather than full-length antibodies could be favorable because of a reduced size and chain number [13]. An scFv with 30 kDa in size has been autodisplayed on *E. coli* cells for translocation studies and drug delivery [134, 135]. By autodisplaying peptides or antibodies, an affinity protein layer with all proteins showing the same orientation can be produced by immobilizing the C-terminal end of the proteins. Flow cytometry screening can be used to sort single *E. coli* cells with autodisplayed affinity peptides or antibodies and high affinity binder can be selected [136]. After incubation of single colonies of sorted *E. coli* cells, the OM layer can be coated to form an immunoaffinity layer on solid matter including a biosensor surface. On this immunoaffinity layer, highly sensitive immunoassays and biosensors without capture antibody treatment can be developed exploiting the orientation and density control as described being an

immanent feature of the autodisplay technology.

As described above, autodisplay technology has advantages for the expression of affinity proteins and enzymes. However, autodisplay has the limitation that it can be only used in gram-negative bacteria such as *E. coli*. *E. coli* grows fast with 20 min of doubling time, so it can used used for a rapid production of OM vesicles. Since *E. coli* is a prokaryote, limited resources for post-translational modifications [110]. Autodisplay of advanced and complex targets such as eukaryotic proteins cannot assure the activity in case post-translational glycosylation is required. For the autodisplay of such proteins needed for outer membrane coating, additional enzymes e.g. glycosyltransferases could be applied. Another disadvantage of autodisplay is, that it is not possible to express proteins that contain integral transmembrane α-helices. This will lead to a misguiding during transport and to a complete degradation of the fusion protein in the periplasm. Therefore interesting human drug targets, such as G-protein coupled receptors (GPCRs) are excluded for being used in the outer membrane coating technology. In general, any signature that could direct the protein to a certain cellular compartment could interfere with autodisplay and hence the passenger protein needs to be carefully checked on such signals before being used in the autodisplay based outer membrane coating approach.

A major disadvantage of the technology as presented here, using outer membrane coating of solid matters for orientation and density control of proteins, is that solid matter and outer membrane vesicles are connected to each other by hydrophobic interactions i.e. relatively week forces. This can lead to a distortion of the analytical sandwich layer by the fluid containing the analyte, in case there is a continuous high or abrupt flow. It can lead as well to bleeding effects in case outer membrane vesicles are used for the coating of C-18 RP particles as the stationary phase in column chromatography [137]. In this respect a covalent coupling of the outer membrane with surface displayed proteins would be preferable [113]. It could be achieved e.g. by incorporation of a non-canonic amino acid in a loop of the β-barrel at the inner side of the outer membrane in a bio-orthogonal approach [138]. Subsequently click-chemistry would be applied to couple the unnatural amino acid, e.g. *p*-azido-phenylalanine, to a functional group of the solid matter. This is under current investigation.

**7. Conclusions**

In conclusion, the sensitivities of immunoassays and biosensors have been improved by orientation and density

control as mechanism based effect of the autodisplay of proteins and using OM preparations of such cells for coating solid matters. Autodisplay technology can easily express target proteins on the OM of *E. coli* with all protein molecules directed in the same orientation by using DNA recombinant methods. *Escherichia coli*-based immunoassays can detect an analyte in a complex biological matrix such as bovine serum and human serum, and thus can be applied for analytical and medical diagnosis. An OM with autodisplayed proteins can be isolated using detergents such as Triton X-100 and the OM preparation can be used to coat solid supports through simple dipping methods. The immunoaffinity layer based on the OM with autodisplayed affinity proteins used for coating has improved the sensitivity of immunoassays and biosensors. Based on these studies, autodisplay of other affinity proteins or peptides and antibody libraries can be developed for highly sensitive medical diagnosis.

**Acknowledgements**

This research was supported by the National Research Foundation of Korea (NRF-2016R1D1A3B03934169), the Korean Health Technology, R&D project, Ministry of Health & Welfare (HI14C0559), and the Hallym University Research Fund, 2016 (HRF-201603-006).

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**Figure legends**

Fig. 1. The structures of (a) Z-domains autodisplay and (b) autodisplaying vector and its expression mechanism.

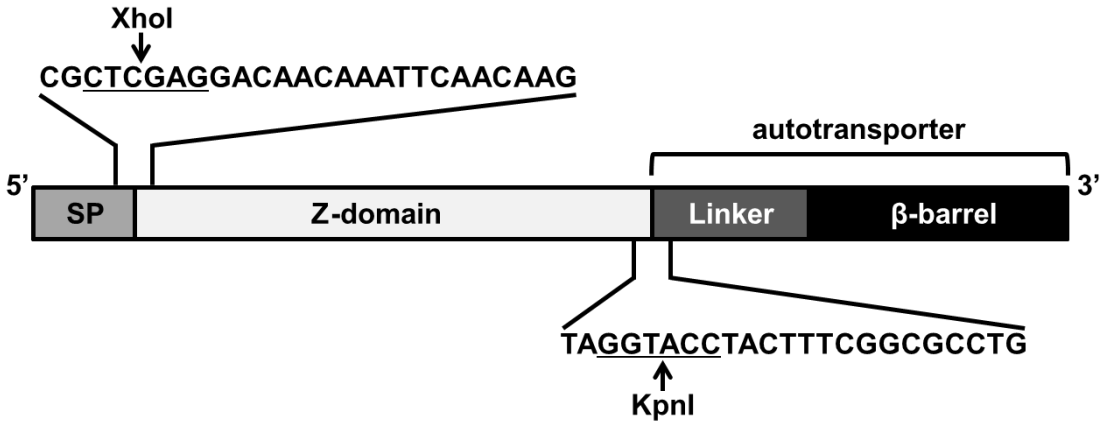
Fig. 2. (a) The structure of isolated E. coli vesicle with autodisplayed affinity protein and (b) the orientation and density control by autodisplayed affinity proteins.

Fig. 3. Properties of the coated OM layer. (a) The activity of OM layer by changing the hydrophobicity of surface [62]. (b) Thickness of the OM layer by different methods [87]. (c) cyclic voltammetry (CV) and (c) Nyquist plot of the OM coated gold electrode [61]. For the CV measurement, redox couple of 5 mM ferricyanide solution was measured with 10 mV/s of scan rate using saturated calomel electrode as the reference electrode. Nyquist plot was obtained at the frequency range with 10 – $10^6$ Hz. And 50 mV of potential amplitude.

Fig. 4. AFM images of the OM layer during the sequential treatment of antibodies [61].

Fig. 5. SPR response of the formation of the immunoaffinity layer by the sequential treatment of the isolated OM vesicle and capture antibodies [66].

Fig. 1.(a)




(b)

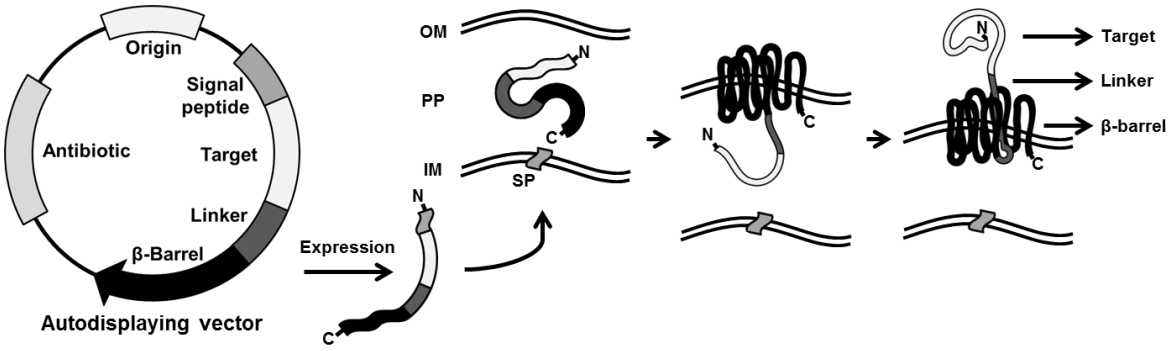
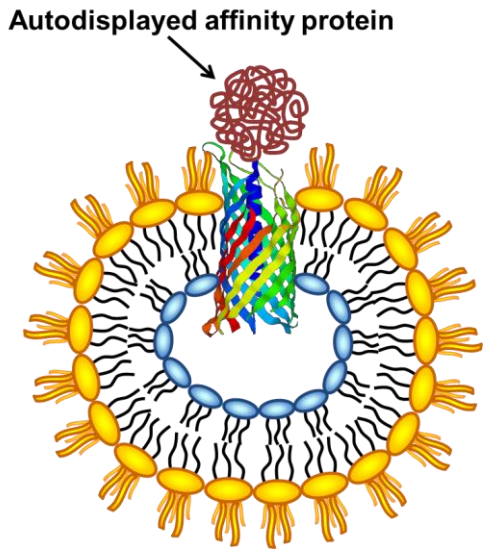


Fig. 2.

(a)

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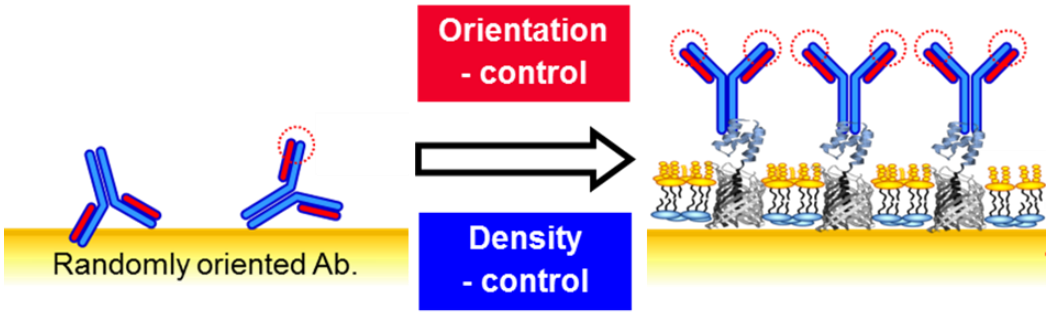
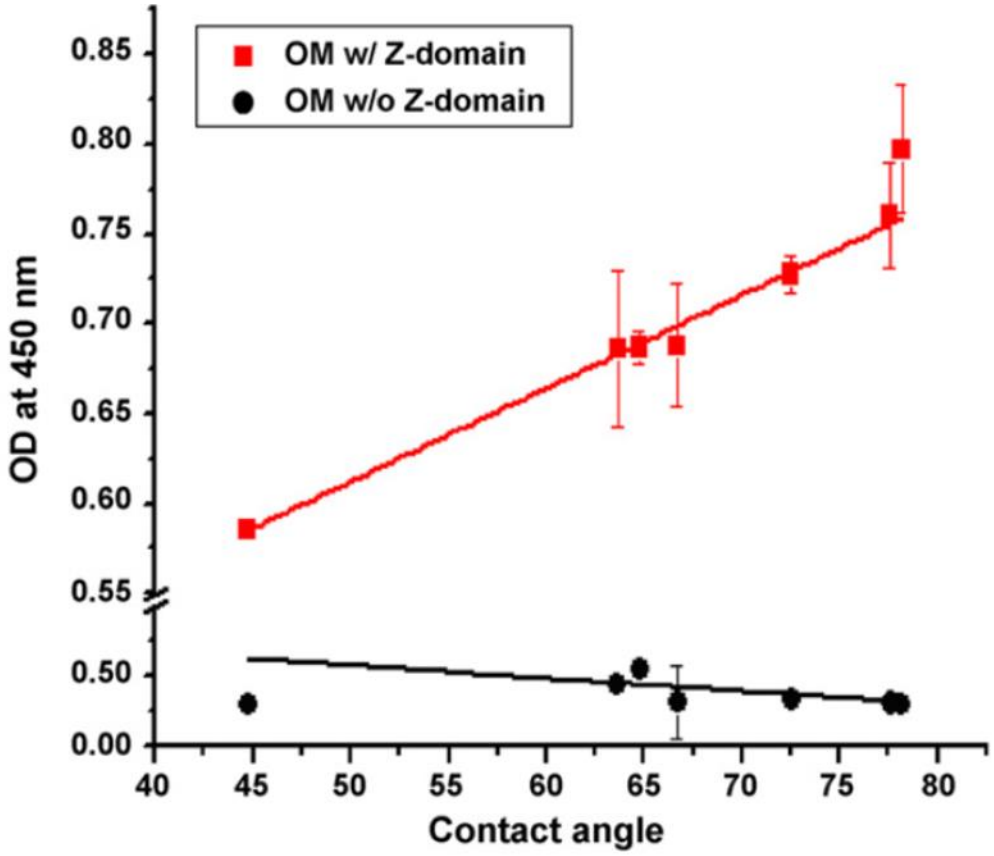


Fig. 3.

(a)

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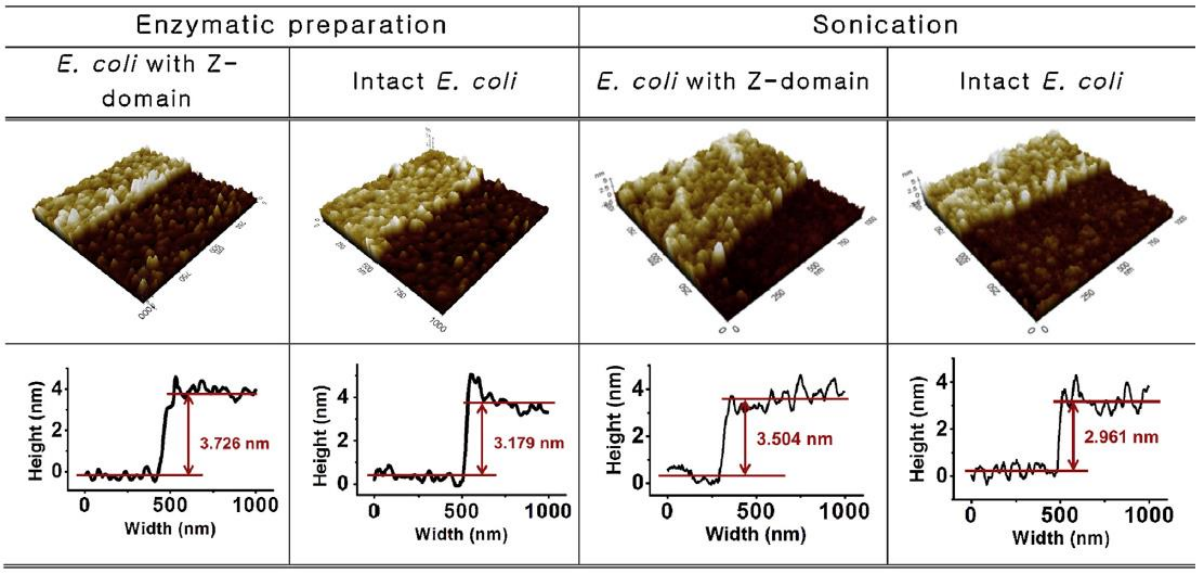

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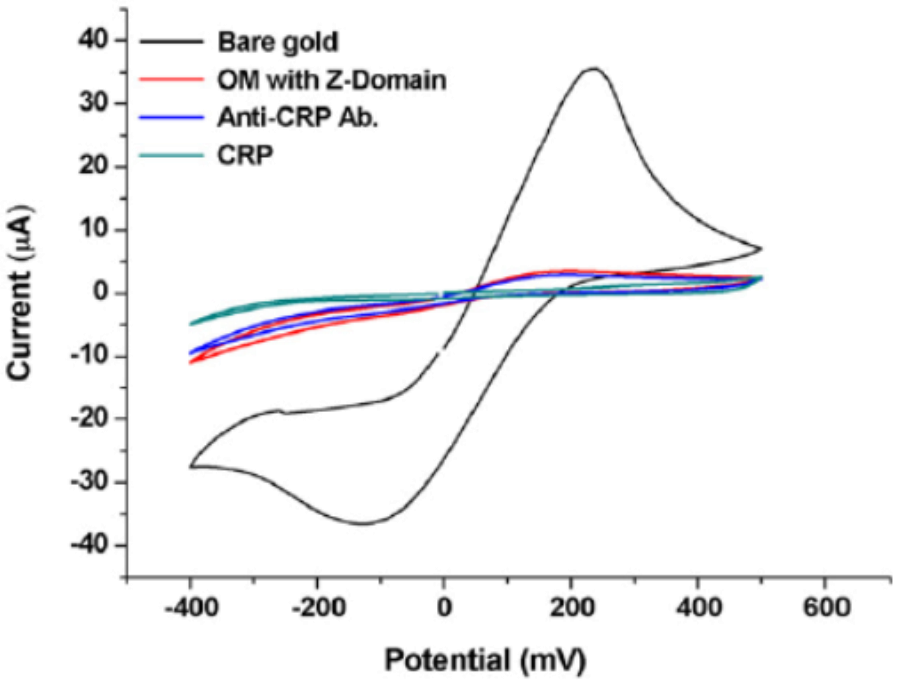


(d)

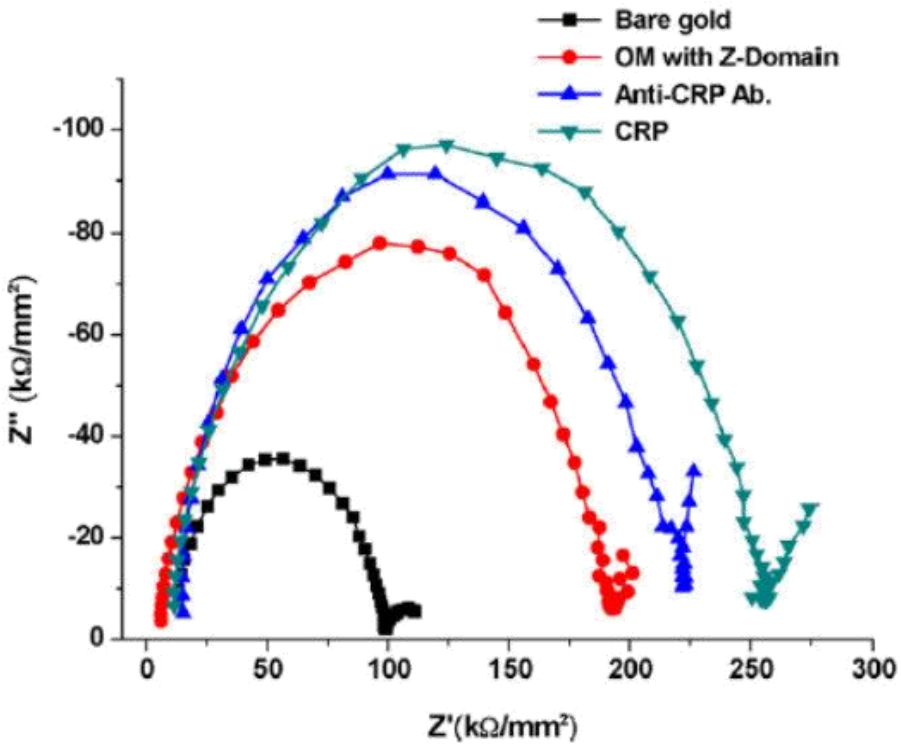

Fig. 4.

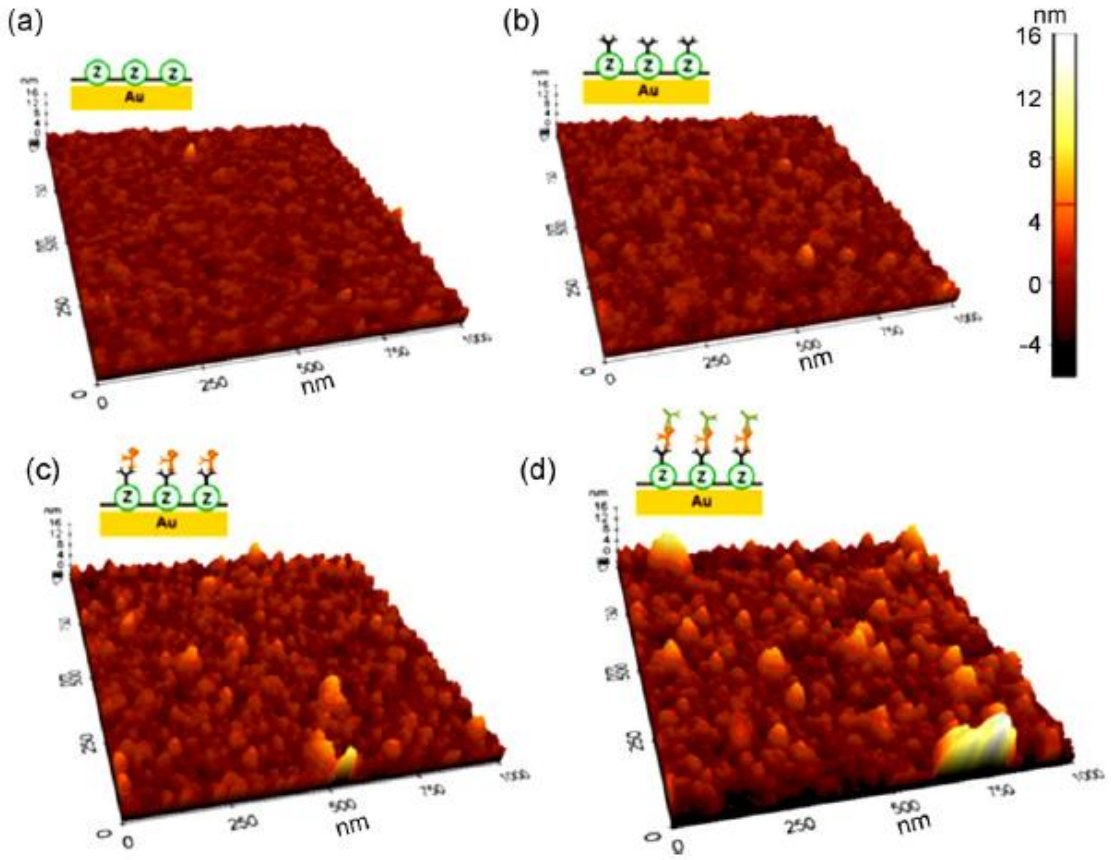

Fig. 5.

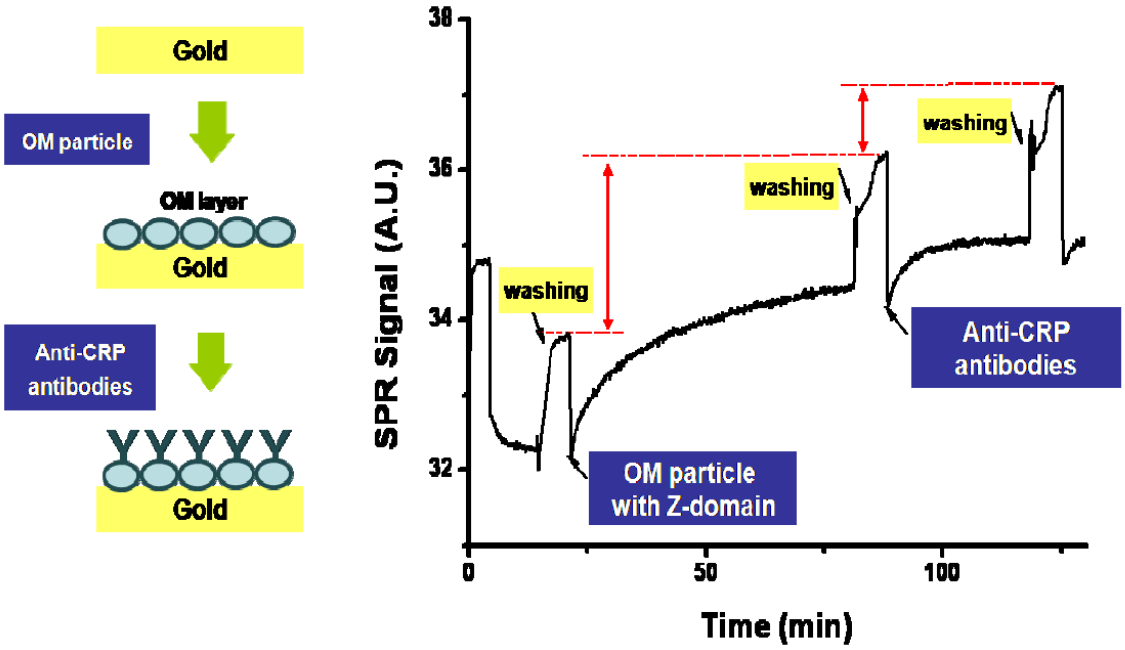

Table 1. Selected marker proteins for purity assay of outer membrane isolation.

| *E. coli* compartment | Marker for western blot | Marker for enzymatic assay |
|---|---|---|
| Outer membrane | OmpA [59] | KDO [93] |
| Periplasm | β-lactamase [94] | β-lactamase [95] |
| Inner membrane | SecA [96] | NADH oxidase [97] |
| cytoplasm | β-galactosidase [98] | β-galactosidase [98] |

Table 2. Autodisplayed targets, type of assays, analytes, and limit of detections (LOD) of autodisplay technology-based immunoassays and biosensors.

| Autodisplayed target | Assay | Analyte | LOD (ng/mL) | Comparison assay | LOD (ng/mL) | Ref. |
|---|---|---|---|---|---|---|
| Z-domain | Whole cell homogeneous immunoassay | CRP | 1 | ELISA | 10 | [67] |
| | Flow cytometry-based immunoassay | Troponin-I | 9 | Protein A beads | 60 | [72] |
| | Multiplexed immunoassay | HBsAg | 25 | - | - | [112] |
| | | CRP | 6 | | | |
| | Whole cell coated microplate immunoassay | CRP | 0.5 | Physical adsorption | 10 | [113] |
| | Magnetic bead-based immunoassay | CRP | < 1 | Unmodified magnetic bead | 100 | [114] |
| | OM coated microplate-based immunoassay | CRP | 1.5 | Physical adsorption | 25 | [62] |
| | OM coated SPR biosensor | CRP | < 100 | Physical adsorption | > 10,000 | [66] |
| | OM coated SPR biosensor | Human IgG | < 1 | Physical adsorption | 10 | [61] |
| Streptavidin | OM coated microplate-based assay | Biotinylated HRP | 1 | Physical adsorption | 300 | [60] |