



Development of an immunosensor using oriented immobilized anti-OmpW for sensitive detection of *Vibrio cholerae* by surface plasmon resonance

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ABSTRACT

The first SPR sensor for detection of bacteria was reported in 1998 with high detection limit as much as 10^7 cfu/mL. Since then, a lot of effort has been made to lower detection limit and increase sensitivity of detection mainly by using of different assay formats, immobilization strategies, suitable antibodies, minimizing non-specific adsorption and improving the quality of SPR devices. The aim of this paper is to introduce the potential of an antibody against recombinant outer membrane protein (anti-OmpW) in sensitive detection of *Vibrio cholerae* by developing an immunosensor based on SPR and compare the sensitivity of this method with former report for detection of *V. cholerae* published in 2006. Recombinant OmpW antigen (a bacterial outer-membrane protein) of *V. cholerae* was expressed and purified and raising of polyclonal rabbit anti-OmpW was done. Protein G was covalently immobilized on 11-MUA SAM via amine coupling and bioaffinity-based oriented immobilization of anti-OmpW was done on protein G layer. The results showed high affinity interaction between OmpW and anti-OmpW ($K_D = 2.4 \times 10^{-9}$ M) and the detection limit of fabricated immunosensor was 43 cells/mL. The apparent reasons for achieving this low LOD are discussed.

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1. Introduction

Vibrio cholerae (causative agent of cholera illness), is a member of list of bacterial, viral, protozoan, and helminth waterborne pathogens introduced by World Health Organization (WHO) (Connelly and Baeumner, 2012) that cause an acute intestinal disease with watery diarrhoea, high dehydration, vomiting, acidosis, and circulation disorders (Nandi et al., 2005). It is estimated that 3–5 million cases and more than 100,000 deaths occur each year worldwide (Zhang and Gou, 2014).

Detection of pathogens in rapid, sensitive, and specific way is the first and most important step in effective disease control (Shi et al., 2012). Over the years, many researches have focused on the development of biosensors rather than conventional microbiological methods (Ahmed et al., 2014; Singh et al., 2014).

The first application of surface plasmon resonance (SPR) biosensor for immunosensing was proposed in 1983 by Liedberg, Nylander, and Lundström, as a result of multidisciplinary

researches in Laboratory of Applied Physics, Linköping Institute of Technology, under the program of “applications of physics in chemistry, biology and medicine” (Liedberg et al., 1983, 1995; Lundström 2014). Since then SPR is known as a very sensitive, label-free and real time optical biosensor and powerful method for studying biomolecular interactions (Homola, 2008; Nikfarjam et al., 2016). SPR has been successfully used for the rapid, sensitive, and quantitative detection of different pathogens since 1998 (Abadian et al., 2014; Frataamico et al., 1998).

Using bioaffinity linker molecules provide the orientation-controlled immobilization so that the paratopes of the antibodies are exposed to the surface (Dudak and Boyaci, 2009). In this study, protein G was covalently immobilized on 11-mercaptopundecanoic acid (11-MUA) self-assembled monolayer (SAM) via amine coupling and bioaffinity-based oriented immobilization of an antibody against recombinant outer membrane protein (anti-OmpW) of *Vibrio cholerae* was done on protein G layer and the detection of different concentration of OmpW antigen and *V. cholerae* was performed. The detection of *Vibrio cholerae* O1 by SPR spectroscopy using a monoclonal antibody against lipopolysaccharide (LPS) antigen of *Vibrio cholerae* with lower detection limit of 10^5 cells/mL was reported before by Jyoung et al. (2006). The aim of

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this short communication is mainly to discuss the differences between two assays for detection of *V. cholerae*.

2. Materials and method

2.1. Chemicals and reagents

N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 11-MUA, protein G, and sodium acetate were supplied by Sigma-Aldrich. Ethanolamine was purchased from Fluka. All chemicals and reagents used were of analytical grade. All solutions were prepared using deionized water throughout the experiment and all buffers for SPR tests were filtered through 0.22 μm filter and degassed prior to use.

2.2. Microorganisms

Vibrio cholerae O₁ serovar Ogawa, was obtained from microorganisms bank, Iranian Biological Resource Center (IBRC), Iran. Bacterial suspensions were prepared by optical density measurement. Absorbance of 0.257 is equal to bacterial suspension of 3×10^8 cells/mL (equivalent to McFarland Standard no. 1). Bacteria were adjusted to 10^8 cells/mL and serial dilutions up to 10 cells/mL were prepared.

2.3. Instrumentation

The biomolecular interactions were investigated using a two-channel cuvette-based SPR instrument with an auto sampler incorporated surface plasmon resonance system (Auto lab ESPRIT, Metrohm Autolab, Utrecht, The Netherlands). Channel#1 was the test channel in which sample solutions were added; Channel#2 was used to run reference measurements to which blank or control solutions were added. Commercial sensing surface, the bare gold disks, which covered by 5 nm of titanium as an adhesion layer and a sensing layer of 50 nm of gold, were supplied by Ssens bv, (The Netherlands). The outcome of the SPR measurement was automatically monitored using a PC with data acquisition software version 4.3.1. All kinetic data were obtained using kinetic evaluation software version 5.4 (KE Instruments, The Netherlands).

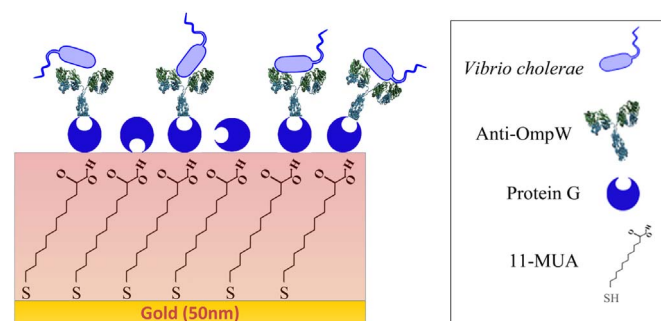
2.4. Production and purification of OmpW antigen and polyclonal anti-OmpW

Recombinant OmpW antigen (MW. 26 kDa) was expressed and purified in our establishment and polyclonal rabbit anti-OmpW was produced and purified. Details are explained in the [Supplementary text 1](#).

2.5. Oriented immobilization of *V. cholerae* antibodies using protein G/antibody complex

After cleaning of gold disk surface with “piranha” solution, it was incubated in 1 mM 11-MUA in ethanol for overnight. The disk was washed 3 times with absolute ethanol to remove excess alkanethiols and three additional times with high-purity water to remove the alcohol and dried under nitrogen.

After the formation of 11-MUA SAM, the gold sensor disk was inserted in the SPR machine. A two-step immobilization technique was done and 25 $\mu\text{g/mL}$ protein G was first bound covalently to the 11-MUA SAM surface with routine amine coupling method (Fischer, 2010), which in turn, then bioaffinity capturing of antibodies were done by injecting 25 $\mu\text{g/mL}$ anti-OmpW in second step to sensor chip in SPR cuvette to form protein G/antibody complex.



Scheme 1. Schematic illustration of oriented immobilization of anti-OmpW on sensor chip and sensing of *V. cholerae*. (Not drawn to scale).

Although immobilization of protein G on SAM layer with the amine coupling procedure is random and some portion of protein G immobilized such a conformation that cannot bind to antibodies, this can provide more degree of freedom for immobilized antibodies to bind to its related antigen. An overview of oriented immobilization of antibodies using protein G and sensing of *V. cholerae* is illustrated in [Scheme 1](#).

2.6. Biosensing protocol

The anti-OmpW immobilized on the sensor chip was used for the sensing of different concentrations of OmpW antigen (0.08–5 $\mu\text{g/mL}$) and *V. cholerae* (10^1 to 10^5 cells/mL in steps of 10^1) by direct assay. For biomolecular interaction and affinity measurements of OmpW antigen with immobilized anti-OmpW antibody, 50 μL of the sample solution containing a selected concentration of OmpW antigen in the HEPES buffer was injected in both channels from the 384 well microtiter plate and then association was performed for 900 s and dissociation was performed for 600 s. The surface was regenerated to baseline position by aspirating/dispersing regeneration buffer (15 mM NaOH+0.2% SDS) in the cuvettes for 10 min after each concentration interaction.

The affinity interactions between antigen and immobilized antibody were calculated using the equilibrium dissociation constant (K_D). K_D and maximum binding capacity of analyte (B_{max}) values were evaluated using the “kinetic evaluation software ver. 5.4”. The data were fitted using a simple 1:1 langmuir fit model.

3. Results and discussion

Immobilization of antibodies to the sensor surface is one of the most important steps in SPR assay that determine the success of biosensor (Skottrup et al., 2008). Providing a good degree of freedom for the antibodies on the sensor surface and improving the sensitivity and selectivity are the results of oriented immobilization of antibodies on the sensor surface so that recognition sites are not sterically hindered (Dudak and Boyaci, 2009; Skottrup et al., 2008). In this study, we have investigated detection of *V. cholerae* by a SPR biosensor, and the obtained results are shown below. Moreover, we will discuss the differences between this study and that of Jyoung et al. for the detection of *V. cholerae* O1 (Jyoung et al., 2006).

3.1. Production and purification of OmpW antigen

The results of expression and purification of OmpW protein are presented in [Supplementary text 2](#) and [Fig. S1](#).

In the current diagnostics, antibodies are key affinity ligands, but they need to have low cross-reactivity. Thus, antibodies should be produced against a single target molecule that is exposed to

surface and constitutively expressed in the target species only (Leonard et al., 2003). OmpW is one of these species-specific antigens rationally selected by our team for production of antibodies with high target specificity. In a study, the test results of enzyme-linked immunosorbent assay (ELISA) and dot-ELISA for anti-OMP antisera of *V. cholerae* showed very high reactivity and specificity (Martinez-Govea et al., 2001). In another study in our establishment, the ability of monoclonal and polyclonal antibodies against in house produced recombinant OmpW in specific diagnosis of *V. cholerae* by an agglutination test was demonstrated (Fasihi, 2014). These results motivated us to evaluate the potential of anti-OmpW in detection of *V. cholerae* by SPR method. Polyclonal antibodies (pAbs) are preferred, because a purified pAb produced against a specific antigen, recognize more than one epitope on a target molecule, that generally provide more robust detection and is anticipated to have a higher average affinity due to the multivalent nature and they are more tolerant of minor antigen changes (George, 2000; Subramanian et al., 2006). In contrast, Jyoung et al. have used monoclonal antibodies (mAbs) against lipopolysaccharide (LPS) antigen of *V. cholerae* O1 for the detection of the target bacteria (Jyoung et al., 2006). Mabs have some drawbacks including lower average affinity and very narrow specificity than pAbs (Damodaran, 1997), costly and long time production procedure as extraction of LPS from bacterial strains are time-consuming in comparison to production of recombinant proteins such as OmpW, and instability of mAbs to environmental stresses can limit their long term storage and their field applicability (Tawil et al., 2012). Moreover, bacterial polysaccharides are poor immunogens due to their structural similarities with glycolipids and glycoproteins in comparison to protein antigens such as OmpW (Weintraub, 2003). Mabs against LPS have narrow detection range for *V. cholerae* serogroups, and therefore they are not suitable for detection of cholera outbreaks (Chatterjee and Chaudhuri, 2003).

3.2. SPR characterization for interaction of OmpW antigen with the immobilized anti-OmpW

The interaction of various concentrations of OmpW with its related antibody on 11-MUA SAM was conducted to assess the

sensing performance. As shown in Fig. 1, the SPR sensorgram angle shifts exhibit concentration dependant at different concentrations of OmpW antigen from 3 to 200 nM.

The logarithmic plot of the protein concentration versus SPR signal was linear from 3 to 50 nM of the target protein. The calculated limit of detection (LOD) was 0.3 nM using the equation $LOD = 3.3 \sigma/s$ (Shrivastava and Gupta, 2011), where σ is the standard deviation of 10 repeated Rmax of the blank response (the signal of HEPES buffer on the nanobiosensor) and s is the slope of the calibration curve.

The hydroxyl groups of ethanolamine that was used in amine coupling method for blocking of unreacted NHS-ester groups play an important role in preventing non-specific binding to surface chip (Frederix et al., 2004). In the study of Jyoung et al. (2006), the hydrophobic alkyl chains of hexanethiol that act as spacer molecules on mixed SAM, have lesser effect on non-specific binding and this perhaps had an influence on high LOD of their study.

Using the “kinetic evaluation software ver. 5.4”, kinetic parameters, such as K_D and B_{max} values were calculated and were found to be 2.4 nM and 124.4 m°, respectively for binding of OmpW antigen with immobilized antibody. This low K_D value (If $K_D < 10$ nM, then it represents the high affinity interactions) (Sikarwar et al., 2014) represents high affinity interaction of the OmpW antigen with the immobilized antibody.

Surface coverage of antibodies on sensor chip is important to obtain desired analyte binding response. The recommended ligand concentration for immobilization by Biacore is 1–100 µg/mL (Healthcare, 2008; Laure Jason-Moller and Bruno, 2006) and in most researches, the concentration of ligand fall into this range (Stojanović et al. 2014; Subramanian et al., 2006). In another work of our team, maximum interaction response of anti-OmpW immobilized on 11-MUA SAM and *V. cholerae* was achieved at 25 µg/mL of antibody concentration (data not shown). This concentration is very far from concentration of 50 pmol/mL (7.5 ng/mL) Mab against *V. cholerae* O1 in Jyoung et al. study (Jyoung et al., 2006) and this low antibody concentration could eventuated in the poor surface coverage whereupon the LOD, though the incubation time of antibody to self-assembled protein G layer in their study was 2 h. Eminency of their work was that the

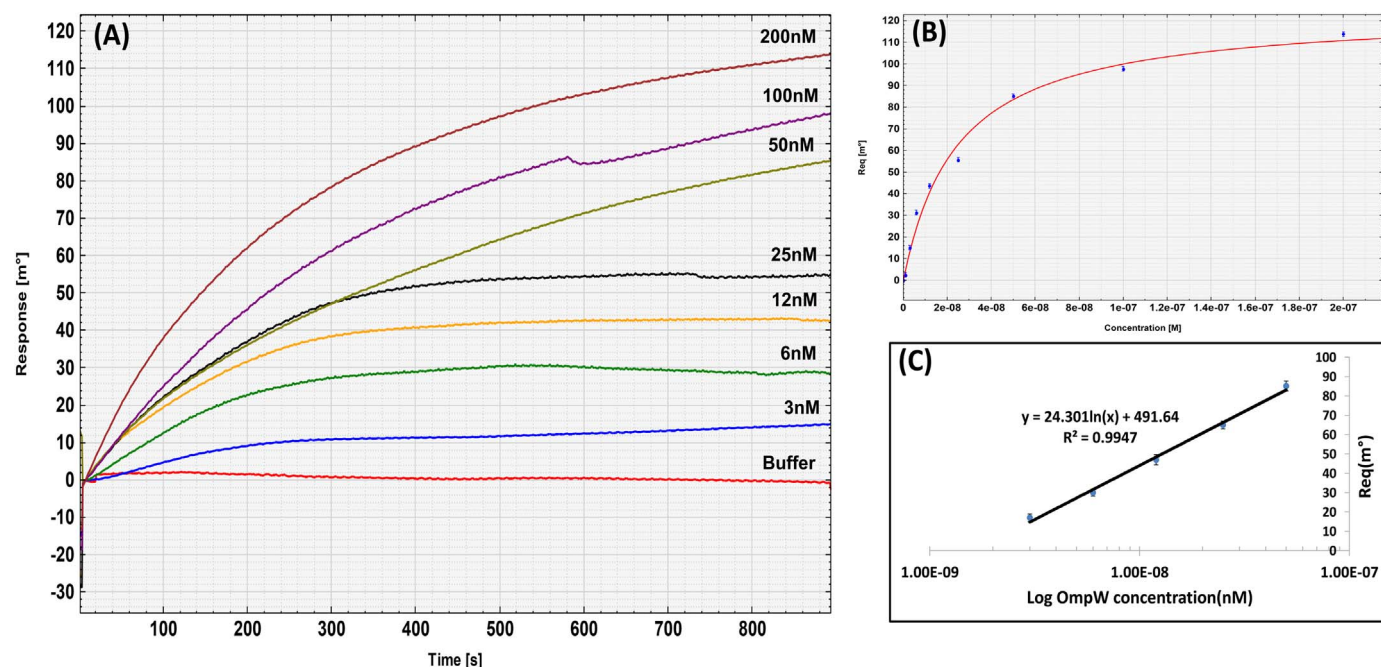


Fig. 1. (A) SPR sensor response overlay plot for the interaction of different concentrations (3–200 nM) of OmpW with 25 µg/mL immobilized anti-OmpW. (B) Langmuir isotherm plot of equilibrium angle (Req) versus OmpW concentration. (C) Logarithmic plot of equilibrium angle (Req) versus OmpW concentration.

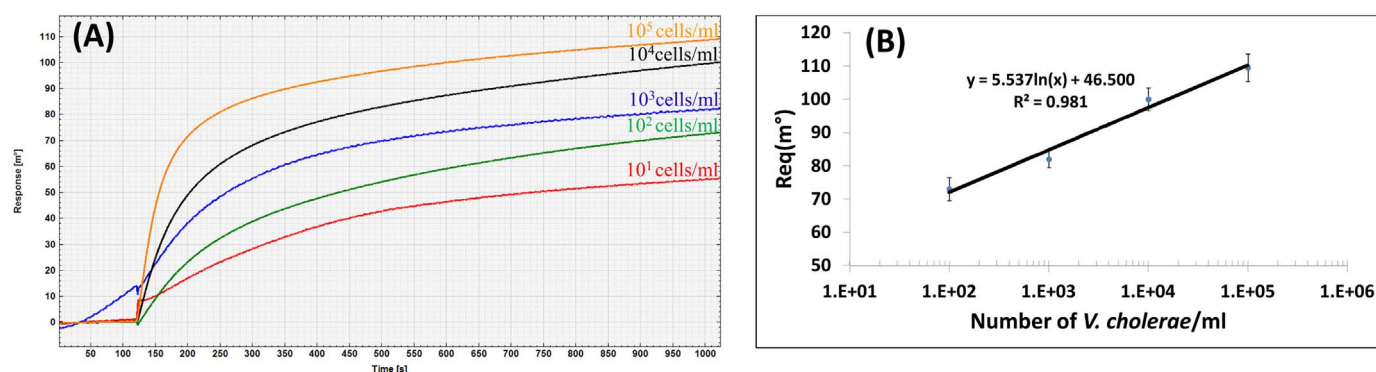


Fig. 2. (A) Overlay interaction of 10–10⁵ cells/mL *V. cholerae* with oriented immobilized anti-OmpW on protein G. (B) Log scale calibration curve of the assay.

molar ratio of mixed SAM was 2:1 of 11-MUA and hexanethiol, otherwise if the molar ratio of carboxylated thiol was as low as 1:40 as in Puttharugsa et al. (2011), or 5:95 as in Bonroy et al. study (Bonroy et al., 2006), the surface coverage of antibody would be insufficient to generate detectable response.

3.3. Interaction of *V. cholerae* with oriented immobilized anti-OmpW using protein G/antibody complex

Protein G specifically and securely binds the Fc region of the antibodies, with an orientation that allows the fragment antigen-binding (Fab) region of the antibodies to be freely available for efficient binding to antigen. This important feature provides an important advantage over other immobilization techniques (Makaraviciute and Ramanaviciene, 2013). After the surface preparation, various concentrations of *V. cholerae* were injected to the SPR system. Fig. 2 shows the response overlay plot for the sample solutions with different concentrations of *V. cholerae* (10¹ to 10⁵ cells/mL).

The results showed that the resonance angle shift is linearly proportional to the log of bacterial suspension in concentrations ranging from 1×10^2 to 1×10^5 cells/mL ($R^2 = 0.981$). From the experiment, the LOD of the sensor was found to be 43 cells/mL for *V. cholerae* using the equation explained in Section 3.2.

The LOD in the SPR experiment depends on several factors, including the binding affinity of analyte-ligand molecules (Nguyen et al., 2015). Low K_D value (2.4×10^{-9} M) was calculated for interaction of OmpW and anti-OmpW resulted in specific binding of *V. cholerae* to sensor surface with LOD of 43 cells/mL. This result is significant, because we did not use any pre-treatment and/or pre-concentration of samples. The most significant advantage of bacterial detection with minimum sample preparation or signal enhancement steps is the rapidity of the assay that is vital for detection of highly contagious disease.

There are a lot of variable factors that can impact on LOD determination, such as sample treatment (using live, heat killed, ultrasonicated, and lysed bacteria) (Taylor et al., 2005), accuracy of SPR device used for detection, type of fluid handling (flow cell or cuvette) (Schasfoort and Tudos, 2008), affinity of antibodies to target analyte (Liang et al., 2007), purity and surface coverage of immobilized antibodies (Nguyen et al., 2015), type of antibody immobilization (physical adsorption, chemical conjugation or using linker molecules) (Torun et al., 2012), random or oriented immobilization of molecular recognition element (MRE) (Baniukovic et al., 2013), type of samples (bacterial culture, environmental or food samples), running time, regeneration condition, etc.

Using a cuvette-based rather than flow cell-based SPR sensor helped us to achieve this acceptable LOD, as in continual flow systems, mass transport of analytes (specially trapping of large and

massive analytes, such as bacterial cells) into matrices can limit assay sensitivity (Barlen et al., 2007; Rusmini et al., 2007).

To sum up, it is our firm belief that high affinity interaction between OmpW surface antigen and its polyclonal antibody plays major role to achieve more sensitive detection of *V. cholerae* than the study of Jyoung et al. in 2006 (Jyoung et al., 2006). Other subsidiary issues were: using a cuvette-based rather than flow cell-based SPR sensor SPR, device accuracy (professional two channel SPR device, Autolab ESPRIT, with angle resolution < 0.02 m° instead of Multiskop™, a versatile modular setup which incorporates ellipsometry, surface plasmon spectroscopy, waveguide modes, their corresponding imaging techniques and Brewster angle microscopy in a single instrument, with angle resolution of 1 m° (Harke et al., 1997)), reduced non-specific binding on the sensor surface by changing the unreacted carboxyl groups to hydrophilic hydroxyl groups (OH) in comparison to hydrophobic methyl (CH₃) in hexanethiol spacer on mixed SAM, using optimized antibody concentration (25 µg/mL) to achieve adequate surface coverage of antibodies rather than 7.5 ng/mL that they used.

The results of real sample analysis of the developed SPR immunosensor confirmed its application in wastewater samples, although its recovery percent was about 84%. (data not shown).

4. Conclusions

In this study, we have described a SPR-based immunosensor for detection of *V. cholerae*, using bioaffinity-based oriented immobilization of antibodies against *V. cholerae* surface antigen (anti-OmpW). Our work reveals high affinity interaction between OmpW and its antibody ($K_D = 2.4 \times 10^{-9}$ M), resulted in sensitive detection of *V. cholerae* with LOD of 43 cells/mL. By comparing of our results to the previously published SPR immunosensor for *V. cholerae* detection developed by Jyoung et al. in 2006, we concluded that the high affinity interaction between OmpW surface antigen and its polyclonal antibody had an important role in enhancing the sensitivity of the immunosensor. In addition, some other factors including application of a cuvette-based rather than flow cell-based SPR, accuracy of the device (Autolab ESPRIT with angle resolution < 0.02 m° instead of Multiskop™ with angle resolution of 1 m°), reducing non-specific adsorption on the sensor surface, and achieving adequate surface coverage of antibodies on sensor chip, were clearly helped out the proposed method to reach much higher sensitivity. It should be considered as a drawback of this method that in multiple analyses, harsh regeneration buffer condition could desorb antibodies from protein G. Therefore, the effect of a subsequent crosslinking step between affinity ligand (protein G) and antibodies could be investigated in future researches.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2016.07.006>.

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