

Detection of Classical Swine Fever Virus by a Surface Plasmon Resonance Assay

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Abstract

Test sera from vaccinated pigs at different time-frame were used to assess the sensitivity and specificity of an optimized Surface Plasmon Resonance (SPR)-chip-based detection method. Biomolecular interaction between Classical Swine Fever Virus (CSFV) and serum antibody were investigated in relative time using Biacore SPR system. An amount of 8860.93 RU of CSFV in 10mM sodium acetate of pH 5.0 was fixed on CM5 dextran matrix sensor chip. Serum from vaccinated animals was allowed to run over the whole CSFV in triplicate. The relative response from serum of 5th week and 7th week old swine towards the immobilized CSFV reacted variably. The 10 fold diluted serum response unit of 94.24 ± 11.34 RU indicated a limited immune response at week 5. However in week 7, the highest response in the serum at same dilution demonstrated a 2 fold increase at 189.33 ± 2.57 RU. Regeneration with glycine-HCl at pH 2.0 enabled successful baseline reversion after each analysis. The herein established, whole virus immobilized SPR-chip could serve as a prototype for a rapid and sensitive CSFV diagnosis assay.

Keywords: SPR technology; Chip-based viral assay; Virus; Hyper immune serum

Abbreviations: CpMV: Cowpea Mosaic Virus; CSFV: Classical Swine Fever Virus; ELISA: Enzyme-linked Immune Sorbent Assays; hCMV: Human Cytomegalo Virus; HRV3: Human Rhinovirus type 3; IEF: Isoelectric Focusing; IgG: Immunoglobulin G; Mab: Monoclonal antibody; SPR: Surface Plasmon Resonance; TMV: Tobacco Mosaic Virus; RU: Response Unit

Introduction

Classical swine fever disease, a major concern in the swine-related populations, is caused by classical swine fever virus (CSFV) which belongs to the same flavivirus family of other human pathogens, namely Dengue fever virus, Yellow fever virus, Japanese encephalitis virus, and Tick-borne encephalitis virus [1-3]. CSFV is an enveloped RNA virus, with a diameter of 40-60 nm and consisting of 12.3 kb with a genome size poly protein coding for 3898 amino acids [4]. CSFV causes classical swine fever (CSF) characterized by acute haemorrhagic disease, thrombocytopenia and immunosuppression. In less virulent strains of CSFV long-term persistence and inherited disease to offspring can occur [5].

Commonly CSFV antigens could be traced from crude or impure samples with low concentrations of viruses by fluorescent antibody and ELISA [6]. For convenience Risatti and co-workers developed a portable real-time RT PCR assay for in situ CSFV detection [4]. The development of multiplex real-time RT PCR (RT-MRT-PCR) offers dual purpose, CSFV detection and genotyping [7], and for the quantitative and also differential detection of wild-type viruses from C-strain of CSFV in vaccinated swine herds [8]. Although PCR-based detection is fast and convenient, it relatively needs stable viral genome [9]. Problems may arise from high mutation rates which lead to false positive results [10]. To date, the application of Loop-Mediated Isothermal Amplification (LAMP) assay has been claimed to be rapid, simple and able to distinguish CSFV to other porcine viruses [11]. Yin and co-workers have demonstrated an interesting study to detect E2 gene sequence of CSFV using reverse-transcriptase LAMP (RT-LAMP), declaring that it is cost-effective, convenient and a vital aid to unequipped clinical laboratories [12]. However, LAMP assay requires high purity of extracted RNA and deflects cross-contamination that leads to false positive [13].

The label-free Surface-Plasmon-Resonance (SPR) technology has been reported for use in classical swine fever disease diagnostics to monitor real-time biomolecular interaction [14]. CSFV immunogenic glycoproteins such as E2 and E1/Erns are useful for detection, prevention and vaccination. SPR application using CSFV E2 protein, revealed good correlation with ELISA measurements of E2 antibody titer in swine sera [14]. However, the use of whole CSFV immobilized chip would be a practical and wholesome approach in designing CSFV diagnosis assay as it mimics the *in vivo* system [15]. Immobilization of whole viruses coated sensor chip using SPR technology is not new. Whole virus immobilization has been reported for human cytomegalovirus (hCMV) [16], adenovirus [17], Human Rhinovirus Serotypes 3 (HRV3) [18], Tobacco Mosaic Virus (TMV) and cowpea mosaic virus (CpMV) [19]. Herein, the present study described the optimization process of whole CSFV immobilization on CM5 dextran sensor chip for subsequent investigation on real-time interaction of whole CSFV-serum antibody.

Materials and Methods

Samples collection

Purified CSFV was prepared according to established method [20]. The virus was referred as ligand since it was immobilized on the CM5 sensor chip. The blood samples were collected from immunized animal (PESTIFFA[®]-modified live vaccine against classical swine fever Chinese strain CL) aged between 5 to 7 weeks old. Blood samples were collected via plain tube through jugular. Serums were separated and stored at -70°C.

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SPR detection system

SPR analysis was performed using Biacore 3000 (Uppsala, Sweden) and Research Grade Sensor chip CM5, at an assay temperature of 25°C. HBS-EP buffer (10 mM HEPES pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20) was used as the running buffer in the experiment.

Immobilization pH scouting

A series of sodium acetate ranging from pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 were prepared. Sodium acetate (10 mM) works well for many proteins as recommended by BIA core. CSFV was diluted in each solution and pH scouting test was performed to find the appropriate immobilization buffer. The virus solutions were placed in 4°C overnight to allow them settled homogeneously prior to use. Before determining the pH range, the isoelectric point (pI value) for CSFV must be known. In this case, the pI is 8.3 which had been retrieved from ExPASy Protein Analysis System from ExPASy website. It is a server of Swiss Institute of Bioinformatics which computes theoretical isoelectric point (pI) and molecular weight from the uploaded sequence from user or from its database. Efficient pre concentration is between pKa of sensor chip and pI of ligand ($pK_a < \text{immobilization pH} < pI$ of ligand). Herein, pKa refers to equilibrium constants of the dextran matrix of CM5 sensor chip. The pKa of dextran matrix is 3.5 and the pI of CSFV is 8.3. Thus, it is hypothesized that the suitable pH for CSFV immobilization lies in between those ranges.

Immobilization of whole virus

Virus was diluted at 1:10 (v/v) in 10 mM sodium acetate of pH 5.0 and prepared in 1 ml. It was then covalently coupled to a sensor chip CM5 via primary amine groups. The procedure for immobilization was followed accordingly. HBS-EP was allowed to run at constant flow rate of 5 μ L/min. Then the sensor surface was activated with 1:1 N-hydroxysuccinimide (NHS 115 mg/ml) and N-ethyl-N'-dimethyl amino propyl carbodiimide (EDC 115 mg/ml) solution. A constant rate of 20 μ L/min virus was injected into the flow cell for about 16 minutes. Finally 1 M ethanolamine pH 8.5 was used for deactivation of excess reactive groups.

Binding and regeneration analysis

Serum of 5 and 7 weeks old vaccinated swines were diluted at 1:10, 1:100 and 1:1000 in HBS-EP buffer and prepared in 1 ml of total volume. The analytes were injected over the immobilized virus at the rate of 2 μ L/min on the sensor chip with HBS-EP as the transport buffer. The signal in Response Unit (RU) was displayed in sensorgram. The time taken for binding analysis was around 50 seconds. The regeneration of the sensor chip surface was performed by injection of 5 μ L/min glycine-HCl of pH 2.0 for 30 seconds. It was controlled by comparing baseline-resonance units before and after the regeneration procedure.

Scanning electron microscopy (SEM) of the sensor chip surface

To confirm the immobilization of virus particles on the sensor chip, the virus image was captured through SEM [17]. Immediately after immobilization, the CM5 sensor chip was undocked from the machine and disassembled. The CSFV immobilized-gold-film was carefully removed and fixed for 5 minutes with 1.5% glutaraldehyde in phosphate buffer pH 7. Then it was stained for 15 minutes with 1% aqueous uranyl acetate. After washing with distilled water, the chip was allowed to dry. A stub was placed at the glass surface beneath the dextran layer functioning as a holder throughout the analysis. The

chip was sputter-coated with a thin layer of gold (Baltec SCD005) for 2 minutes. The images of the virus particles were observed and captured at 30.0 kV accelerating voltage using SEM Philips XL30 ESEM.

Results

Maximum binding capacity

The pH scouting for immobilization was done to find the appropriate immobilization pH (pre concentration buffer). A series of sodium acetate with pH ranging from 4.0 up to 5.5 was chosen. The PI was determined to be 8.3 as described in the materials and methods. Based on the result in Figure 1, pH 5.0 showed the highest RU of 28802.07, at which the maximum saturation had been achieved. It also reflects the maximum rate of interaction and covalent coupling of the pre-soaked virus to the dextran matrix.

Direct immobilization of whole CSFV

Figure 2 depicted the whole process of immobilization that at the end successfully immobilized 8860.93 RU of CSFV. The result was presented using BIA evaluation software version 4.1. Arrows indicate: (1) target for immobilization level, (2) injection of running buffer, (3) activation of sensor chip's surface with amine coupling method, (4) injection of CSFV, and (5) deactivation of excess reactive surface with ethanolamine. (6) amount of immobilized CSFV: 8860.93 RU. For a general carboxy methylated dextran-coated chip CM5, 1000 RU represents 1 ng/nm² [21]. Thus in this case, about 8.86 ng/nm² of CSFV in 10 mM of sodium acetate of pH 5.0 had been immobilized (Figures 1 and 2).

Interaction analysis between virus and antibody

Interaction analysis in Figures 3a and 3b demonstrated the ability of serum antibody of 5 and 7 weeks old swine to recognize and bind to the immobilized virus, respectively. The serum antibody of 7 weeks old swine could recognize and bind to the immobilized virus at higher RU level (189.33 RU) in comparison to the serum antibody of 5th week old swine at 10-fold dilution (94.24 RU). Sensogram on the top was the interaction between the serums of 10-fold dilution, followed by 100- and 1000-fold dilution. From here, the highest RU reading was declared i.e. the top sensogram. The result herein is the typical result for any binding interaction occurred in the flow cell of the sensor chip (Figure 3).

Validation of the established chip

Table 1 presented the relative response of serum antibody towards immobilized CSFV in 10-fold serial dilution factors up to 1000-fold dilution. Statistics and the linearity test were performed using Microsoft Office Excel version 2003. Life span of the reused chip was determined by subtracting the baseline results just before the first binding analysis took place and immediately at the end of the final cycle. Through regeneration scouting procedure, glycine at pH 2.0 was fairly good to remove the serum from the immobilized virus (data not shown) and useful to dissociate the virus-antibody complexes without giving much disruption to the immobilized virus. For three consecutive repeats, consistent readings were achieved, indicating efficient usage of the regeneration buffer to break up the binding to analytes whilst retaining the immobilized CSFV. Up to 18 successive cycles were performed in the present study and slightly 0.58% of RU increment was counted at the end of the experiment. The reactivity of serum antibody towards the immobilized CSFV was carried out using a series of different sample dilutions which is 1:10, 1:100 and 1:1000 (v/v) in order to test the linearity of the assay [22]. The linear regressions for the reactivity

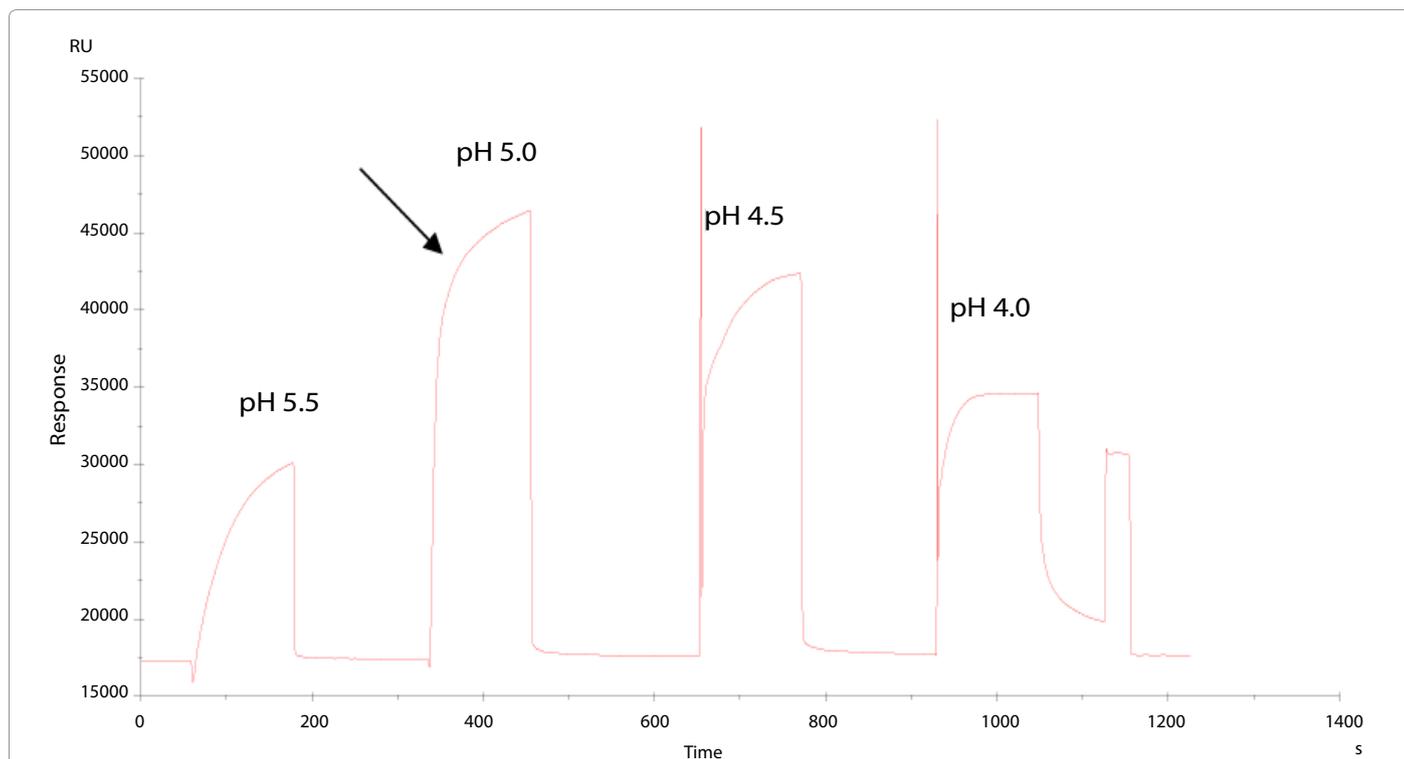


Figure 1: pH scouting for whole CSFV immobilization. Pre-immobilization of whole CSFV was tested for maximum binding capacity in each pre-immobilization buffers at pH ranging from 4.0 to 5.5, when run over the carboxymethylated matrix of CM5 sensor chip. The optimum pH for pre-immobilization is at pH 5.0 as pointed by arrow.

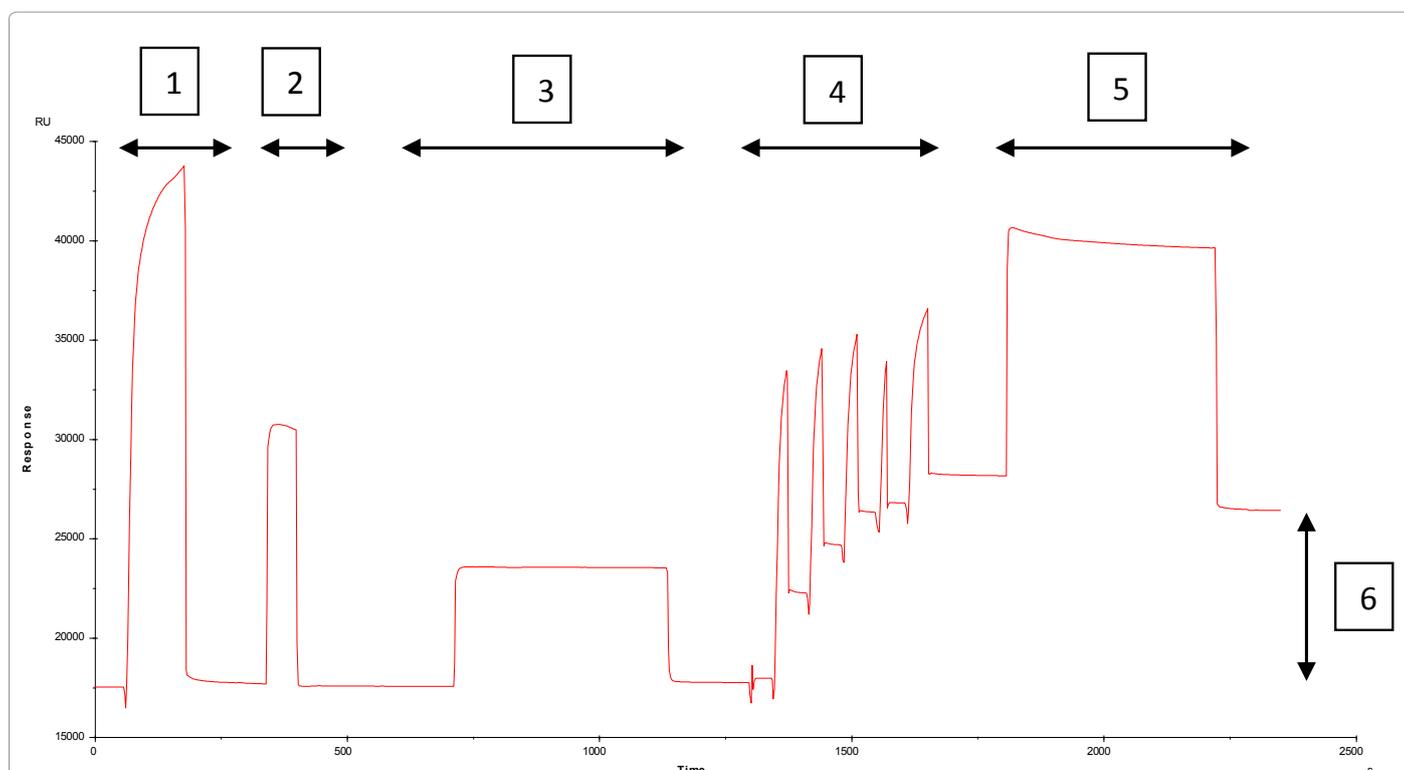


Figure 2: Immobilization of CSFV on CM5 sensor chip. Immobilization of CSFV in 10 mM Sodium acetate at pH 5.0 in flow cell 4 with reported response unit of 8860.93 RU. Result was presented using BIA evaluation software version 4.1. Arrows indicate: (1) target for immobilization level, (2) injection of running buffer, (3) activation of sensor chip's surface with amine coupling method, (4) injection of CSFV, (5) deactivation of excess reactive surface with ethanolamine, and (6) amount of immobilized CSFV. The activation and non-activation event of the reference flow cell was not shown.

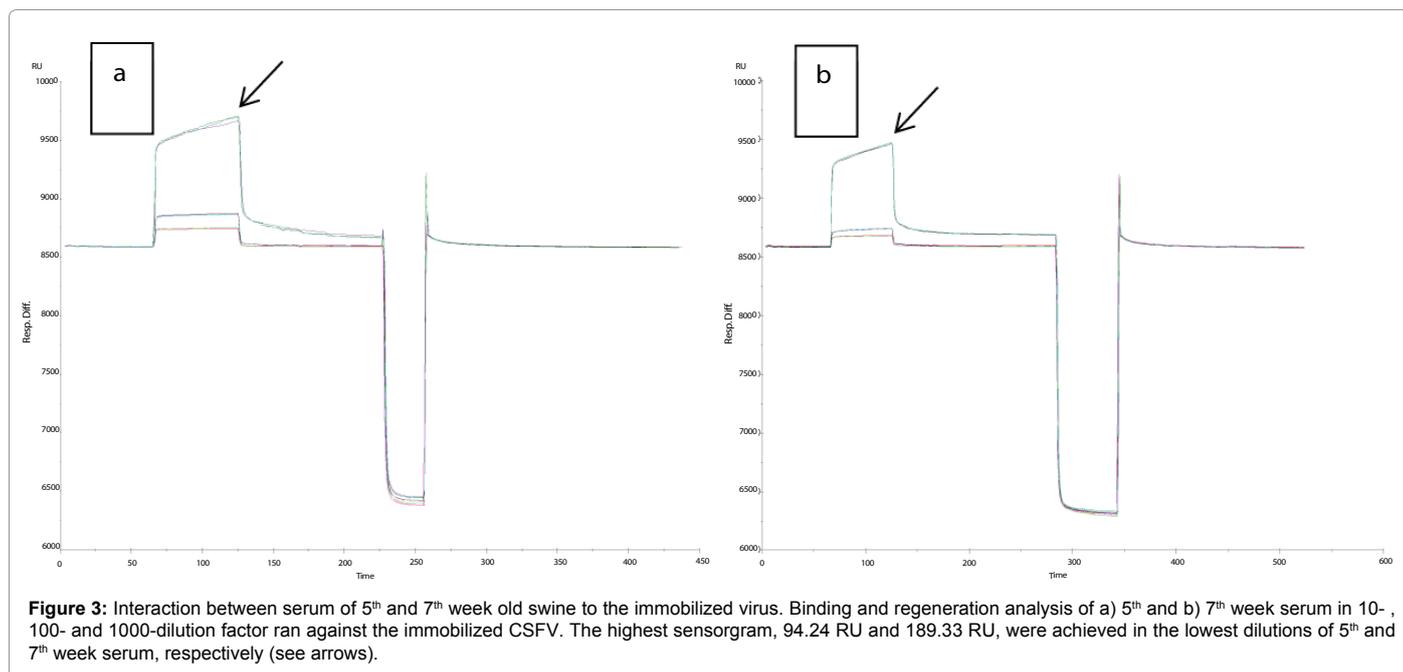


Figure 3: Interaction between serum of 5th and 7th week old swine to the immobilized virus. Binding and regeneration analysis of a) 5th and b) 7th week serum in 10-, 100- and 1000-dilution factor ran against the immobilized CSFV. The highest sensorgram, 94.24 RU and 189.33 RU, were achieved in the lowest dilutions of 5th and 7th week serum, respectively (see arrows).

Serum in HBS-EP buffer	Mean ± S.D. 5 weeks old (RU)	Mean ± S.D. 7 weeks old (RU)
1:10	94.24 ± 11.34	189.33 ± 2.57
1:100	9.43 ± 0.37	27.89 ± 0.19
1:1000	0.15 ± 0.25	11.50 ± 2.19

Table 1: Binding reactivity of immunized serum to the immobilized CSFV. Readings are average value of triplicate readings. The working dilution factor referred to serum that has been serially diluted in HBS-EP buffer for analysis purpose.

of 5th and 7th week serum were 0.8132 and 0.8099, respectively. The findings suggested the use of 1:10 dilution factor rather than 1:1000, as the latter provided almost undetectable data and generated RU that is almost equivalent to the reference flow cell (Table 1).

CSFV distribution on the surface of sensor chip

SEM micrograph presented the intact immobilized CSFV captured under 25000X magnifications (Figure 4a). The icosahedral shape of the virus could be visualized. Viruses were intact onto the surface even after multiple regeneration cycle. The size of CSFV on SEM seemed larger (100 μm) than its regular size (60 nm), a logical effect on gold-coated virus particle. Maintaining the integrity of immobilized virus is crucial especially for enveloped virus such as CSFV because the presence of detergents may destroy the liposomes in the lipid membrane of the enveloped virus [23]. Nonetheless, preserving the phenotypic conformation of non-enveloped virus is also important as detergents may interrupt the virus-antibody binding [17]. Figure 4b showed the blank flow cell under 5000X magnifications (Figure 4).

Discussion

Although the approach on whole virus immobilization on sensor chip began two decades ago, undeniably not all viruses had been successfully studied for this purpose. Enveloped viruses establish a contact region at the cell surface, which is stabilized by the formation of receptor-ligand complexes. Whole virus immobilization acts in a similar manner as the *in vivo* system where the virus adsorbed on the cell receptor and the antibodies in blood serum flows over them [15]. In reality, favorable contact energy stemming from the formation of the ligand-receptor complexes in the interaction zone is sufficient to drive the engulfment of the virus by the cell [24]. Immobilization of

ligand (CSFV) bound to an antibody through the formation of multiple noncovalent bonds formed ligand - analyte complex. The binding affinity (strength) is actually produced from the summation of the attractive and repulsive forces of vander Waals interactions, hydrogen bonds, salt bridges and hydrophobic force [25]. Current methods to detect CSFV antibodies are such as the Enzyme Linked Immunosorbent Assay (ELISA) and immune chromatographic strip. Unlike the study herein, the most antigens used were the CSFV glycoprotein instead of the whole CSFV. Besides, SPR has additional features to analyze the binding interaction between molecules in real-time and has potential to detect targeted antibody in a single chip depending on the experimental design [26,27].

Initial immobilization with plant viruses i.e. tobacco mosaic virus (TMV) and cowpea mosaic virus (CpMV) was on CM5 dextran layers in formic acid buffers at pH 3 and 4, respectively [19]. The conformational integrity of TMV and CpMV has been revealed by using specific mabs for screening viable epitope. Casasnovas and Springer identified 10mM of sodium acetate at pH 5.7 to be suitable for immobilizing non enveloped human rhinovirus serotypes 3 (HRV3) on CM5 sensor chip [18]. By increasing the molarity of sodium acetate (20 mM) to an acidic pH of 3.5, non-enveloped adenovirus can be immobilized on similar chip [17]. Therefore, pH condition in SPR has considerable impact in virus-antibody interaction. pH 5.0 demonstrated as the best immobilization condition for CSFV with its pI 8.3. Being an enveloped virus, CSFV is stable at pH 5.0 to pH 10.0 in normal environment and is less stable below pH 3.0 or higher than pH 11.0 (<http://www.classicalswinefever.org>, 2009). As reported, human rhinovirus (HRV) was best immobilized using pH 5.7 [18], which fall below its pI value 6.8 [28]. Acidic environment at pH 5.3 effectively allows HRV3 to attach on cell membrane prior penetration and the

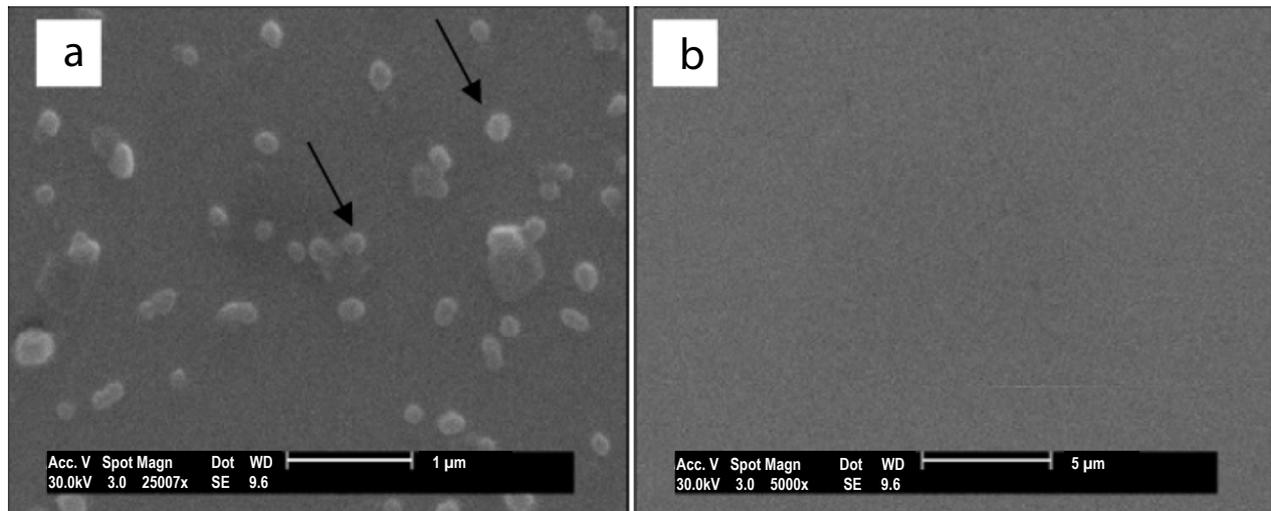


Figure 4: SEM of immobilized CSFV on the dextran surface and empty flow cell. a) Comparison of flow cell 4 consisting gold-coated CSFV and, b) empty flow cell 1 as viewed under SEM.

viral *de novo* synthesis can be detected under similar circumstances [29]. Nurani and co-workers found that an efficient uncoating event of HRV, *in vitro* and *in vivo*, occurred at a mild lower pH of 5.5 to 6.0 [30]. Nonetheless, low pH helps the penetration of virus genome to the receptor-bound on membrane-proximal region of the virus particle during acidification of endosomes [30].

The pH condition for immobilization should be lower than pI value in order to ensure the highest electrostatic attraction of the positively charged virus towards the negatively charged dextran matrix [21]. With pI value of 4.3-7.9 [31], the non enveloped adenovirus can be successfully immobilized in acetate buffer of pH 3.5 [17]. Similar pattern has been demonstrated in CpMV, a nonenveloped virus, where the optimized pH for immobilization was at 4.0 when the pI value was at 4.5 [19,32]. Henceforth, knowing the pI value, which relates to the electrophoretic mobility of viruses, could assist in determining pH range for optimization; minimize sample usage and by-pass additional screening for isoelectric focusing (IEF) [23]. Without having pI information, a complete procedure of pH scouting in various ranges of pre-immobilization buffers would be required.

The amount of immobilized ligand generally depicts the maximum binding capacity that a sensor chip's surface can hold. Therefore, virus size and shape may influence the occupancy level on a type of chip. The rod-shaped TMV of 300x18 nm in size allows immobilization up to 4600 RU, indicating an approximate obtainable RU for a large virus on CM5 dextran matrix [19]. Due to the rod shape of TMV, the 3-D organization of the virus on the activated dextran may be affected and open to many unoccupied areas. This explained in accordance to the detection principle in Biacore; where it measures the changes in refractive index (RI) which is related to changes in mass close to the sensor surface. In an optimized approach for an icosahedral ADV with 60-90 nm diameters in size, virus immobilization achieved at 8322 RU using similar CM5 sensor chip [17]. Similarly, immobilized icosahedral CSFV which is much smaller (60 nm) in comparison to TMV and ADV was immobilized up to 8860 RU. The immobilization of icosahedral HRV of 30 nm in size, at 10000 RU onto CM5 sensor chip, further support the notion that smaller and symmetrical virus could cover activated chip surface more effectively [18]. Biotinylated human influenza virus has been immobilized on streptavidin sensor chip at around 4000 RU [33]. The low RU could possibly due to the

losses of purified virus during separation of biotinylated virus from the unbiotinylated pool. Comparatively, influenza virus is only slightly bigger than ADV (80-120 nm). Therefore, the drastic drop in viral immobilization level pointed out the usage of chip type. Undeniably, the CM3 sensor chip is recommended for larger molecules compare to CM5 sensor chip. Referring to an unpublished data [26], the previous work on *Pseudorabies Virus* (PrV) was done on CM3 sensor chip because of its size range from 200-250 nm. As CSFV similar to Poliovirus and Rhinovirus falls in the range of 30-60 nm, which is almost 5-fold smaller than PrV, thus it was only tested on CM5 sensor chip for this CSFV.

Previously, the binding interaction of monoclonal antibody WH211 as ligand with immobilization level of 9366.4 RU interacted with CSFV at 163.5 RU [27]. Similarly using the same chip and machine, with 8860.93 RU of immobilization of CSFV, the range of RU interaction is expected to be within the range. Tenfold serum dilutions from 7 week old swines averaged a higher response (189.33 ± 2.57 RU) in comparison to the tenfold serum dilutions from 5 week old group (94.24 ± 11.34 RU) (Figures 3a and 3b). On the other hand, the control serum which contains PrV antibodies yields 11.74 ± 0.85 RU. As the in house vaccination of weaned pigs were given at around day 32 of age (4 week plus), this findings was in concordance with previous timing of antibody development at second or third week post-exposure [34].

In summary, the optimized SPR assay based on whole CSFV immobilization can be used as a platform to monitor both virus-antibody binding and periodical assessment of antibody level in vaccinated animals. The approach could be further improvised for a wide range of downstream applications.

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